2-D Proteome Analysis Protocols

Edited by

Andrew J. Link

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METHODS IN MOLECULAR BIOLOGY™

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Andrew J. Link

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Preface

With the completion of sequencing projects and the advancement of analytical tools for protein identification, proteomics—the study of the expressed part of the genome—has become a major region of the burgeoning field of functional genomics. High-resolution 2-D gels can reveal virtually all proteins present in a cell or tissue at any given time, including posttranslationally modified proteins. Changes in the expression and structure of most cellular proteins caused by differentiation or external stimuli can be displayed and eventually identified using 2-D protein gels.

2-D Proteome Analysis Protocols covers all aspects of the use of 2-D protein electrophoresis for the analysis of biological problems. The contributors include many of the leaders in the fields of biochemistry and analytical chemistry who were instrumental in the development of high-resolution 2-D gels, immobilized pH gradients, computer analysis, and mass spectrometry-based protein identification methodologies.

This book is intended as a benchtop manual and guide both for novices to 2-D gels and for those aficionados who wish to try the newer techniques. Any group using protein biochemistry—especially in the fields of molecular biology, biochemistry, microbiology, and cell biology—should find this book eminently useful.

2-D Proteome Analysis Protocols takes the researcher through the complete process of working with 2-D protein gels from making the protein extract to finally identifying the proteins of interest. It includes protocols for generating 2-D protein extracts from most of the standard model organisms, including bacteria, yeast, nematode, *Drosophila*, plants, mouse, and human. This book covers the traditional methods of using carrier ampholytes in the first dimension and the growing movement in the field toward immobilized pH gradients. A brief description of the advantages and disadvantages of each method is given. Analytical and preparative 2-D gels, including the lastest protocols for casting IPG gradients and in-gel rehydration of IPG strips, are covered. For the second dimension, methods for running flatbed or vertical gels, including homogeneous and gradient gels, are given. After running the 2-D gel, there are protocols for protein detection that include autoradiogra-

phy, and Coomassie, silver, and reversible metal chelate stains. *2-D Proteome Analysis Protocols* covers image acquisition of the 2-D gel and has a detailed protocol for computer analysis of the 2-D gel image. With the growing importance of the Internet, this book also includes protocols that enable readers to compare their results with other 2-D databases over the Internet or to construct their own 2-D resolved proteins, focusing on the latest mass spectrometry methods including MALDI-TOF-based peptide mapping, automated tandem mass spectrometry, and nanospray electrospray ionization technology.

Each chapter opens with a description of the protocol and the basic theory behind the method. The Materials section lists all the equipment and reagents required for carrying out the protocol. In the Methods section, each step of the protocol is listed in sufficient detail to successfully execute the experiment. Finally, the Notes section provides invaluable hints and alternatives for dealing with any problems or difficulties that may occur with the protocol.

Many people have contributed time and energy to make *2-D Proteome Analysis Protocols* possible. A special thanks goes to all the contributors, each of whom made many important suggestions and improvements. I wish especially to thank Jenny Fichmann, Reudi Aebersold, Thierry Rabilloud, Mary Lopez, Wayne Patton, Lou Ramagli, Jean-Charles Sanchez, Marc Wilkins, Ron Appel, Angelike Görg, Vitaliano Pallini, and Denis Hochstrasser for invaluable advice and help in preparing this book.

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Contents

Contents

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1

2-D Protein Gel Electrophoresis

An Overview

Jenny Fichmann and Reiner Westermeier

1. Importance

Two-dimensional electrophoresis (2-D) of proteins used to be an art practiced by a few researchers, and their worldwide meetings could be held in a side room of a medium-sized hotel. With the rapidly growing volume of sequence data produced by the genome projects and the development of new mass spectrometry methods, high-resolution protein analysis has become an important tool in molecular biology. High-resolution 2-D can reveal virtually all proteins present in a cell or tissue at any given time, including those with posttranslational modifications and rapid turnover rates. With the new analytical tools and the genomic and protein database networks, large-scale studies can be performed on the actual gene products or proteins, in their precursor, mature, and modified forms. This task has lately been called the proteome project. The proteome projects are the necessary complement to genome analysis, and aim to identify and characterize all proteins expressed by an organism or a tissue *(1)*.

1.1. Definition

The term 2-D protein gel electrophoresis is used in this volume primarily to mean the 2-D electrophoresis technique in which first-dimension isoelectric focusing in a polyacrylamide gel with a pH gradient and a high concentration of urea (**Fig. 1**) is combined with a second-dimension separation on SDS polyacrylamide gels (**Fig. 2**). In the first dimension, the proteins are separated according to their charges (Chapters 14–24a), and in the second dimension, according to their molecular masses (Chapters 25 and 26). The resulting spot
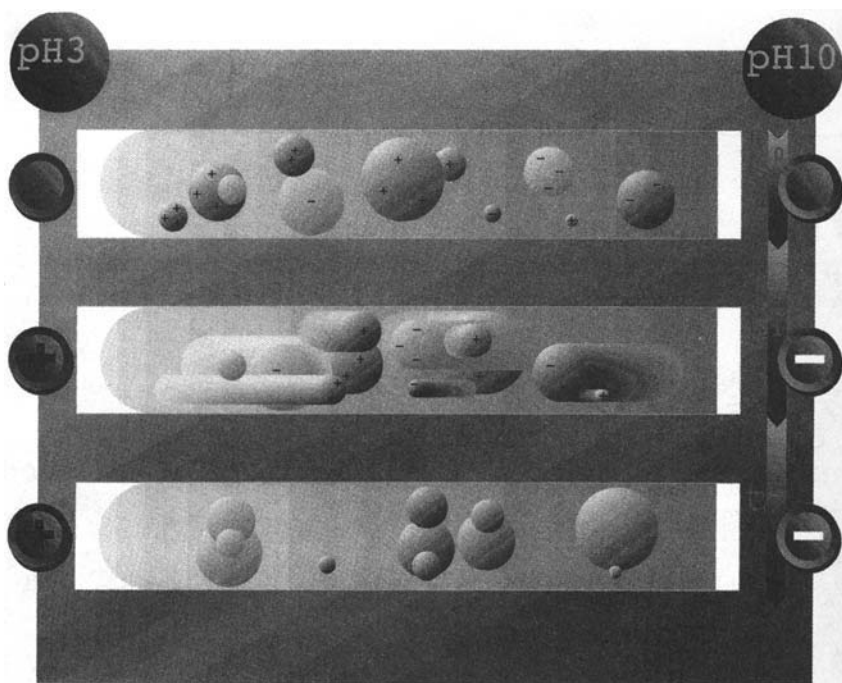
Fig. 1. A schematic showing the isoelectric focusing gel used for the first dimension of 2-D gel electrophoresis. Proteins are being focused to their isoelectric point from time $t_{0 \text{ to } t2}$ using an immobilized pH gradient.

patterns are usually oriented according to the Cartesian convention with the low, acidic isoelectric points to the left and the low molecular weights at the bottom **(Fig. 2)**. Depending on the 2-D application, different gel formats (Chapters 15, 17, 18, 21–24a), reducing or nonreducing conditions (Chapter 27 and 28), different pH ranges (Chapters 16 and 22), and different detection methods can be used (Chapters 31–38).

1.2. What Does the 2-D Separation Method Offer?

Gel electrophoresis has some advantages over other separation techniques. Starting materials, such as cell lysates or tissue extracts, can be applied to gels directly and fractionated with very high resolution. Electrophoretic techniques exhibit minimal loss of hydrophobic protein species. The separated proteins are embedded in the matrix, where they can be detected with very high sensitivity (essentially unlimited exposure time for fluorography, autoradiography, or storage phosphor imager). The isolated proteins can be readily extracted from the matrix for further characterization by sequence analysis or mass spectrometry (Chapters 48–55).
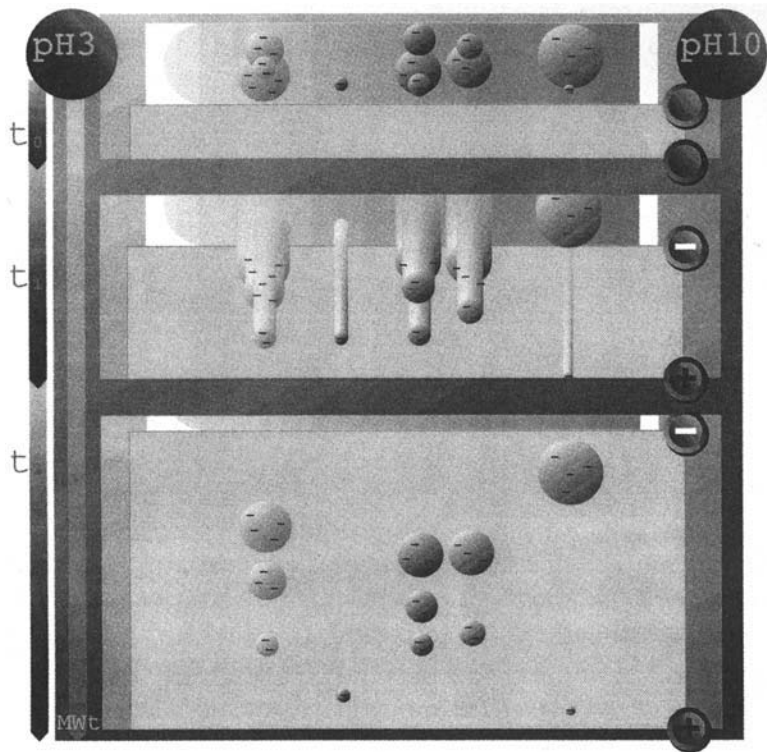
Fig. 2. A schematic showing the SDS-PAGE gel used for the second dimension of 2-D gel electrophoresis. After equilibration, the first-dimension gel is laid on top of a SDS-PAGE gel at time (t_0) and a voltage is applied so that proteins migrates from the 1-D gel into the 2-D gel (t_1). The 2-D gel is run to separate proteins relative to their molecular mass (t_2).

The two separation parameters of 2-D protein gel electrophoresis (isoelectric point and molecular weight) are essentially orthogonal and independent, even though they occur in the same matrix. Either dimension is capable of resolving 100–200 protein species, but the resolving power of the combined techniques is approximately the product of the resolving power of the individual techniques. Up to 10,000 individual protein species have been resolved in a single gel *(2)*, similar in magnitude to the estimated number of expressed proteins in a eukaryotic cell *(3)* or bacterium *(4)*. For studies of minute changes in protein expression or modification in a cell or tissue, it is crucial that the entire array of proteins can be displayed in one gel.

1.3. Technical Aspects

2-D electrophoresis usually proceeds in the following order: perform 1-D isoelectric focusing; exchange the buffer in the focusing gel for the SDS buffer;

place the first-dimension gel in direct contact with the second-dimension SDS gel; perform SDS electrophoresis; detect the separated protein spots. The principle of an isoelectric focusing separation followed by polyacrylamide electrophoresis was first published in 1969 *(5)*. However, a truly successful 2-D method required the development of an effective sample preparation procedure by Klose *(6)*, and O'Farrell *(7)* in 1975.

Because small variations in various steps of this multistep procedure have a major influence on the resulting pattern, mechanization and standardization are essential for reproducible results. The ISO-DALT system for multiple gel casting and running developed by Anderson and Anderson *(8,9)* improved the reproducibility of the technique by addressing a number of mechanical problems in gel casting, loading, and running. Another crucial problem with the earlier versions of the 2-D method was the unsatisfactory performance of the first dimension. The required pH gradient was established by the migration of individual species from complex mixtures of carrier ampholytes to their respective isoelectric points. Batch-to-batch variations of the carrier ampholytes resulted in variations in the shape of the pH gradients. Differences in protein and salt concentrations of the starting material influenced the gradient profile. During focusing, most of the basic gradient is lost in the buffer reservoirs. To visualize the basic proteins, a specialized 1-D gel run under nonequilibrium conditions is required.

Many of these problems are eliminated with the use of immobilized pH gradients (IPG) *(10)*. A pH gradient formed by mixtures of acrylamido buffers is covalently fixed to the acrylamide matrix during gel polymerization. The gradient does not drift and cannot be distorted (Chapters 19–24a). With this improved first dimension, introduced by Görg et al. *(11)*, a substantially wider spectrum of proteins can be resolved throughout the entire pH gradient in one gel. The improved stability and reproducibility of the gradient and commercial availability of precast gradient gels allowed comparisons to be made between gels run in the same lab at different times as well as to gels from outside laboratories run under the same conditions *(12)*.

For the second dimension, SDS gels of different compositions can be used depending on the size of the proteins of interest. For most applications, the discontinuous Tris-HCl/Tris-glycine buffer system of Laemmli is employed. Variations include substituting acetate for HCl to improve gel shelf-life with a pH value below neutrality, and substituting tricine for glycine to improve low-molecular-weight peptide separations. The resolution of the SDS dimension can be further optimized for particular molecular weight ranges by introducing a stacking gel and employing acrylamide concentration gradient gels (Chapters 25 and 26).

1.4. Sample Preparation

Adequate sample treatment is the most important prerequisite for a successful 2-D experiment. The sample preparation procedure must:

1. Stably solubilize all proteins, including hydrophobic species.
2. Prevent protein aggregation and hydrophobic interactions.
3. Remove or thoroughly digest any RNA or DNA.
4. Prevent artifactual oxidation, carbamylation, proteolytic degradation, or conformational alteration.

Each naturally occurring polypeptide should be represented by only one spot in the gel. In general, a cell lysis (or sample solubilization) buffer contains 8–9.8 *M* urea, a nonionic or zwitterionic detergent, carrier ampholytes, dithiothreitol, and, depending on the sample, protease inhibitors and/or protease-free nucleases. There is no universal protocol for sample preparation. Different sample sources require different extraction and lysis techniques (Chapters 2–11).

For autoradiography/fluorography detection, cell proteins must be labeled with radioactive isotopes through growth in the presence of the appropriately radiolabeled precursors. Sample protein concentrations are usually determined before the first dimension (Chapters 12 and 13).

1.5. Protein Load

The amount of proteins applied to a gel can vary between several micrograms to 1 g. If a minor component must be detected against a background of abundant proteins, such as albumin, in a serum sample, a high-protein-capacity system is required. Capacity is dependent on the volume of the gel. Thinner gels provide better sensitivity for most detection methods, and larger and thicker gels offer increased capacity.

Whether the sample should be loaded on the anode or cathode end of the isoelectric focusing gel must be determined experimentally for each new sample. An interesting new approach combines rehydration of a precast dry immobilized pH gradient strip with sample application. The protein sample is mixed with the rehydration buffer and the IPG strip rehydrated in the mixture including the sample. The proteins are distributed over the entire pH gradient. Regardless of where proteins start in the pH gradient, they migrate in the electric field to their corresponding isoelectric points (Chapters 24 and 24a). This approach works particularly well when semipreparative and preparative amounts of sample must be loaded *(13)*.

1.6. Instrumentation

The classical setup for the 2-D technique according to O'Farrell uses vertical thin gel rods for the first dimension and vertical slab gels for the second dimension. With the ISO-DALT system *(8)*, up to 20 gels can be cast and run in parallel. Isoelectric focusing on the immobilized pH gradient gel strips on film supports is more convenient in a horizontal format. The IPG gel strips can be

used with either vertical or horizontal second-dimension SDS gels. Which system should be chosen? Vertical slabs are superior for high protein loads and multiple runs. Horizontal systems employ film-supported gels, which do not change their dimensions during staining and drying, and can be very thin for high resolution and sensitivity of detection. Proteins in very thin gels (<300 μm) can be further analyzed with MALDI-TOF MS directly without intermediate blotting onto a membrane. For good reproducibility and comparability of the results, active temperature control of both dimension runs is very important in both vertical and horizontal formats.

1.7. Detection

With their high sensitivities, autoradiography and fluorography can detect subnanogram amounts of proteins. Gels are exposed on X-ray films (Chapter 31), or they are placed into a storage phosphorimager; the latter method is substantially faster and the images are directly fed to a computer (Chapter 32). Although more quantitative, Coomassie brilliant blue staining (Chapter 34) is gradually being replaced by silver staining, which is about 100-fold more sensitive (Chapter 33).

1.8. Evaluation

The efficient analysis of complete 2-D maps, and the identification and characterization of individual protein spots have only become practically feasible with the latest developments in computer-aided image analysis and access to various 2-D spot and protein and DNA sequence databases (Chapters 39–44) via the Internet. Densitometry, video cameras, or desktop scanners can acquire images. New software has been developed for spot detection and quantitation, spot matching, and pattern subtraction (Chapters 39–43). The utility of public 2-D spot databases requires that the patterns be easily and accurately compared. To eliminate minor displacements, isoelectric point and mol-wt standards must be coelectrophoresed with the sample to aid the matching (Chapter 30).

1.9. Identification and Characterization

The recent development of mass spectrometry techniques for protein analysis has reduced to a few hundred nanograms about one 2-D protein spot, the amount of material required for the identification or further characterization of a protein (Chapters 48–55). MALDI-TOF mass spectrometry can reliably and accurately determine high-resolution masses for macromolecules of >300 kDa (Chapters 50–52). Other widely used identification techniques include comigration of the protein to be identified (Chapter 45); immunoblotting (Chapter 36 and 46); amino acid analysis (Chapter 47); N-terminal sequencing (Chapter 48); and peptide fingerprinting by partial in-gel or eluate digestion followed by SDS-PAGE or reversed-phase HPLC separation (Chapter 45 and 49).

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2

Solubilization of Proteins in 2-D Electrophoresis

An Outline

Thierry Rabilloud

1. Introduction

The solubilization process for 2-D electrophoresis has to achieve four parallel goals:

1. Breaking macromolecular interactions in order to yield separate polypeptide chains: This includes denaturing the proteins to break noncovalent interactions, breaking disulfide bonds, and disrupting noncovalent interactions between proteins and nonproteinaceous compounds, such as lipids or nucleic acids.
2. Preventing any artifactual modification of the polypeptides in the solubilization medium: Ideally, the perfect solubilization medium should freeze all the extracted polypeptides in their exact state prior to solubilization, both in terms of amino acid composition and in terms of posttranslational modifications. This means that all the enzymes able to modify the proteins must be quickly and irreversibly inactivated. Such enzymes include of course proteases, which are the most difficult to inactivate, but also phosphatases, glycosidases, and so forth. In parallel, the solubilization protocol should not expose the polypeptides to conditions in which chemical modifications (e.g., deamidation of Asn and Gln, cleavage of Asp-Pro bonds) may occur.
3. Allowing the easy removal of substances that may interfere with 2-D electrophoresis: In 2-D, proteins are the analytes. Thus, anything in the cell but proteins can be considered an interfering substance. Some cellular compounds (e.g., coenzymes, hormones) are so dilute they go unnoticed. Other compounds (e.g., simple nonreducing sugars) do not interact with proteins or do not interfere with the electrophoretic process. However, many compounds bind to proteins and/or interfere with 2-D, and must be eliminated prior to electrophoresis if their amount exceeds a critical interference threshold. Such compounds mainly include salts, lipids, polysaccharides (including cell walls), and nucleic acids.

4. Keeping proteins in solution during the 2-D electrophoresis process: Although solubilization *stricto sensu* stops at the point where the sample is loaded onto the first-dimension gel, its scope can be extended to the 2-D process, *per se*, since proteins must be kept soluble until the end of the second dimension. Generally speaking, the second dimension is an SDS gel, and very few problems are encountered once the proteins have entered the SDS-PAGE gel. The one main problem is overloading of the major proteins when micropreparative 2-D is carried out, and nothing but scaling up the SDS gel (its thickness and its other dimensions) can counteract overloading an SDS gel. However, severe problems can be encountered in the IEF step. They arise from the fact that IEF must be carried out in low ionic strength conditions and with no manipulation of the polypeptide charge. IEF conditions give problems at three stages:

   a. During the initial solubilization of the sample, important interactions between proteins of widely different p*I*s and/or between proteins and interfering compounds (e.g., nucleic acids) may happen. This yields poor solubilization of some components.

   b. During the entry of the sample in the focusing gel, there is a stacking effect owing to the transition between a liquid phase and a gel phase with a higher friction coefficient. This stacking increases the concentration of proteins and may give rise to precipitation events.

   c. At or very close to the isoelectric point, the solubility of the proteins comes to a minimum. This can be explained by the fact that the net charge comes close to zero, with a concomitant reduction of the electrostatic repulsion between polypeptides. This can also result in protein precipitation or adsorption to the IEF matrix.

Apart from breaking molecular interactions and solubility in the 2-D gel, which are common to all samples, the solubilization problems encountered will greatly vary from one sample type to another owing to wide differences in the amount and nature of interfering substances and/or spurious activities (e.g., proteases). The aim of this outline chapter is not to give detailed protocols for various sample types, and the reader should refer to the chapters of this book dedicated to the type of sample of interest. The author would rather like to concentrate on the solubilization rationale and to describe nonstandard approaches to solubilization problems. A more detailed review on solubilization of proteins for electrophoretic analyses can be found elsewhere *(1)*.

2. Rationale of Solubilization-Breaking Molecular Interactions

Apart from disulfide bridges, the main forces holding proteins together and allowing binding to other compounds are noncovalent interactions. Covalent bonds are encountered mainly between proteins and some coenzymes. The noncovalent interactions are mainly ionic bonds, hydrogen bonds, and "hydrophobic interactions." The basis for "hydrophobic interactions" is in fact the

presence of water. In this very peculiar (hydrogen-bonded, highly polar) solvent, the exposure of nonpolar groups to the solvent is thermodynamically not favored compared to the grouping of these apolar groups together. Indeed, although the van der Waals forces give an equivalent contribution in both configurations, the other forces (mainly hydrogen bonds) are maximized in the latter configuration and disturbed in the former (solvent destruction). Thus, the energy balance is clearly in favor of the collapse of the apolar groups together *(2)*. This explains why hexane and water are not miscible, and also that the lateral chain of apolar amino acids (L, V, I, F, W, Y) pack together and form the hydrophobic cores of the proteins *(3)*. These hydrophobic interactions are also responsible for some protein–protein interactions, and for the binding of lipids and other small apolar molecules to proteins.

The constraints for a good solubilization medium for 2-D electrophoresis are therefore to be able to break ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bridges under conditions compatible with IEF, i.e., with very low amounts of salt or other charged compounds (e.g., ionic detergents).

2.1. Disruption of Disulfide Bridges

Breaking of disulfide bridges is usually achieved by adding to the solubilization medium an excess of a thiol compound. Mercaptoethanol was used in the first 2-D protocols *(4)*, but its use does have drawbacks. Indeed, a portion of the mercaptoethanol will ionize at basic pH, enter the basic part of the IEF gel, and ruin the pH gradient in its alkaline part because of its buffering power *(5)*. Although its pK_a is around 8, dithiothreitol (DTT) is much less prone to this drawback, since it is used at much lower concentrations (usually 50 mM instead of the 700 mM present in 5% mercaptoethanol). However, DTT is still not the perfect reducing agent. Some proteins of very high cysteine content or with cysteines of very high reactivity are not fully reduced by DTT. In these cases, phosphines are very often an effective answer. First, the reaction is stoichiometric, which in turn allows the use of a very low concentration of the reducing agent (a few mM). Second, these reagents are not as sensitive as thiols to dissolved oxygen. The most powerful compound is tributylphosphine, which was the first phosphine used for disulfide reduction in biochemistry *(6)*. However, the reagent is volatile, toxic, has a rather unpleasant odor, and needs an organic solvent to make it water-miscible. In the first uses of the reagent, propanol was used as a carrier solvent at rather high concentrations (50%) *(6)*. It was, however, found that DMSO or DMF was a suitable carrier solvent, which enabled the reduction of proteins by 2 mM tributylphosphine *(7)*. All these drawbacks have disappeared with the introduction of a water-soluble phosphine, *tris* (carboxyethyl) phosphine (available from Pierce, Rockford, IL), for which 1 M aqueous stock solutions can be easily prepared and stored frozen in aliquots.

2.2. Disruption of Noncovalent Interactions

The perfect way to disrupt all types of noncovalent interactions would be the use of a charged compound that disrupts hydrophobic interactions by providing a hydrophobic environment. The hydrophobic residues of the proteins would be dispersed in that environment and not clustered together. This is just the description of SDS, and this explains why SDS has been often used in the first stages of solubilization *(8–11)*. However, SDS is not compatible with IEF and must be removed from the proteins during IEF.

The other way of breaking most noncovalent interactions is the use of a chaotrope. It must be kept in mind that all the noncovalent forces keeping molecules together must be taken into account with a comparative view of the solvent. This means that the final energy of interaction depends on the interaction *per se* and on its effects on the solvent. If the solvent parameters are changed (dielectric constant, hydrogen bond formation, polarizability, and so forth), all the resulting energies of interaction will change. Chaotropes, which alter all the solvent parameters, exert profound effects on all types of interactions. For example, by changing the hydrogen bond structure of the solvent, chaotropes disrupt hydrogen bonds, but also decrease the energy penalty for exposure of apolar groups and therefore favor the dispersion of hydrophobic molecules and the unfolding of the hydrophobic cores of a protein *(12)*. Unfolding the proteins will also greatly decrease ionic bonds between proteins, which are very often not very numerous and highly dependent on the correct positioning of the residues. Since the gross structure of proteins is driven by hydrogen bonds and hydrophobic interactions, chaotropes decrease dramatically ionic interactions both by altering the dielectric constant of the solvent and by denaturing the proteins, so that the residues will no longer be positioned correctly.

Nonionic chaotropes, such as those used in 2-D, however, are unable to disrupt ionic bonds when high-charge densities are present (e.g., histones, nucleic acids) *(13)*. In this case, it is often quite advantageous to modify the pH and to take advantage of the fact that the ionizable groups in proteins are weak acids and bases. For example, increasing the pH to 10.0 or 11.0 will induce most proteins to behave as anions, so that ionic interactions present at pH 7.0 or lower turn into electrostatic repulsion between the molecules, thereby promoting solubilization. The use of a high pH results therefore in dramatically improved solubilizations, with yields very close to what is obtained with SDS *(14)*. The alkaline pH can be obtained either by addition of a few m*M* of potassium carbonate to the urea-detergent-ampholytes solution *(14)*, by the use of alkaline ampholytes *(11)*, or by the use of a spermine-DTT buffer, which allows better extraction of nuclear proteins *(15)*.

For 2-D electrophoresis, the chaotrope of choice is urea. Although urea is less efficient than substituted ureas in breaking hydrophobic interactions *(12)*, it is more efficient in breaking hydrogen bonds, so that its overall solubilization power is greater. However, denaturation by urea induces the exposure of the totality of the proteins hydrophobic residues to the solvent. This increases in turn the potential for hydrophobic interactions, so that urea alone is often not sufficient to quench completely the hydrophobic interactions, especially when lipids are present in the sample. This explains why detergents, which can be viewed as specialized agents for hydrophobic interactions, are almost always included in the urea-based solubilization mixtures for 2-D electrophoresis. Detergents act on hydrophobic interactions by providing a stable dispersion of a hydrophobic medium in the aqueous medium, through the presence of micelles, for example. Therefore, the hydrophobic molecules (e.g., lipids) are no longer collapsed in the aqueous solvent, but will disaggregate in the micelles, provided the amount of detergent is sufficient to ensure maximal dispersion of the hydrophobic molecules. Detergents have polar heads that are able to contract other types of noncovalent bonds (hydrogen bonds, salt bonds for charged heads, and so forth). The action of detergents is the sum of the dispersive effect of the micelles on hydrophobic part of the molecules and the effect of their polar heads on the other types of bonds. This explains why various detergents show very variable effects ranging from a weak and often incomplete delipidation (e.g., Tweens) to a very aggressive action where the exposure of the hydrophobic core in the detergent-containing solvent is no longer energetically unfavored and leads to denaturation (e.g., SDS).

Of course, detergents used for IEF must bear no net electrical charge, and only nonionic and zwitterionic detergents may be used. However, ionic detergents, such as SDS, may be used for the initial solubilization prior to isoelectric focusing in order to increase solubilization and facilitate the removal of interfering compounds. Low amounts of SDS can be tolerated in the subsequent IEF *(10)*, provided that high concentrations of urea *(16)* and nonionic *(10)* or zwitterionic detergents *(17)* be present to ensure complete removal of the SDS from the proteins during IEF. Higher amounts of SDS must be removed prior to IEF, by precipitation *(9)*, for example. It must therefore be kept in mind that SDS will only be useful for solubilization and for sample entry, but will not cure isoelectric precipitation problems.

The use of nonionic or zwitterionic detergents in the presence of urea presents some problems owing to the presence of urea itself. In concentrated urea solutions, urea is not freely dispersed in water, but can form organized channels (*see* **ref. 18**). These channels can bind linear alkyl chains, but not branched or cyclic molecules to form complexes of undefined stoichiometry called inclusion compounds. These complexes are much less soluble than the free

solute, so that precipitation is often induced on formation of the inclusion compounds, precipitation being stronger with increasing alkyl chain length and higher urea concentrations. Consequently, many nonionic or zwitterionic detergents with linear hydrophobic tails *(19,20)* and some ionic ones *(21)* cannot be used in the presence of high concentrations of urea. This limits the choice of detergents mainly to those with nonlinear alkyl tails (e.g., Tritons, Nonidet P-40, CHAPS) or with short alkyl tails (e.g., octyl glucoside), which are unfortunately less efficient in quenching hydrophobic interactions. Sulfobetaine detergents with long linear alkyl tails have, however, received limited applications, since they require low concentrations of urea. Good results have been obtained in certain cases for sparingly soluble proteins *(22–25)*, although this type of protocol seems rather delicate owing to the need for a precise control of all parameters to prevent precipitation.

Apart from the problem of inclusion compounds, the most important problem linked with the use of urea is carbamylation. Urea in water exists in equilibrium with ammonium cyanate, the level of which increases with increasing temperature and pH *(26)*. Cyanate can react with amines to yield substituted urea. In the case of proteins, this reaction takes place with the α-amino group of the N-terminus and the ε-amino groups of lysines. This reaction leads to artifactual charge heterogeneity, N-terminus blocking, and adduct formation detectable in mass spectrometry. Carbamylation should therefore be completely avoided. This can be easily made with some simple precautions. The use of a pure grade of urea (p.a.) decreases the amount of cyanate present in the starting material. Avoidance of high temperatures (never heat urea-containing solutions above 37°C) considerably decreases cyanate formation. In the same trend, urea-containing solutions should be stored frozen (–20°C) to limit cyanate accumulation. Last, but not least, a cyanate scavenger (primary amine) should be added to urea-containing solutions. In the case of isoelectric focusing, carrier ampholytes are perfectly suited for this task. If these precautions are correctly taken, proteins seem to withstand long exposures to urea without carbamylation *(27)*.

3. Solubility During IEF

Additional solubility problems often arise during the IEF at sample entry and solubility at the isoelectric point.

3.1. Solubility During Sample Entry

Sample entry is often quite critical. In most 2-D systems, sample entry in the IEF gel corresponds to a transition between a liquid phase (the sample) and a gel phase of higher friction coefficient. This induces a stacking of the proteins at the sample–gel boundary, which results in very high concentrations of proteins at the application point. These concentrations may exceed the solubility

threshold of some proteins, thereby inducing precipitation and sometimes clogging of the gel, with poor penetration of the bulk of proteins. Such a phenomenon is of course more prominent when high amounts of proteins are loaded onto the IEF gel. The sole simple, but highly efficient remedy to this problem is to include the sample in the IEF gel. This process abolishes the liquid–gel transition and decreases the overall protein concentration, since the volume of the IEF gel is generally much higher than the one of the sample.

This process is, however, rather difficult for tube gels in carrier ampholyte-based IEF. The main difficulty arises from the fact that the thiol compounds used to reduce disulfide bonds during sample preparation are strong inhibitors of acrylamide polymerization, so that conventional samples cannot be used as such. Alkylation of cysteines and of the thiol reagent after reduction could be a solution, but many neutral alkylating agents (e.g., iodoacetamide, *N*-ethyl maleimide) also inhibit acrylamide polymerization. Owing to this situation, most workers describing inclusion of the sample within the IEF gel have worked with nonreduced samples *(28,29)*. Although this presence of disulfide bridges is not optimal, inclusion of the sample within the gel has proven of great, but neglected interest *(28,29)*. It must, however, be pointed out that it is now possible to carry out acrylamide polymerization in an environment where disulfide bridges are reduced. The key is to use 2 m*M* tributylphosphine as the reducing agent in the sample and using tetramethylurea as a carrier solvent. This ensures total reduction of disulfides and is totally compatible with acrylamide polymerization with the standard Temed/persulfate initiator (Rabilloud, unpublished results). This modification should help the experimenters trying sample inclusion within the IEF gel when high amounts of proteins are to be separated by 2-D.

The process of sample inclusion within the IEF gel is, however, much simpler for IPG gels. In this case, rehydration of the dried IPG gel in a solution containing the protein sample is quite convenient and efficient, provided that the gel has a sufficiently open structure to be able to absorb proteins efficiently *(15)*. Coupled with the intrinsic high capacity of IPG gels, this procedure enables easy separation milligram amounts of protein *(15)* (*see also* **Chapter 24**).

3.2. Solubility at the Isoelectric Point

This is usually the second critical point for IEF. The isoelectric point is the pH of minimal solubility, mainly because the protein molecules have no net electrical charge. This abolishes the electrostatic repulsion between protein molecules, which maximizes in turn protein aggregation and precipitation.

The horizontal comet shapes frequently encountered for major proteins and for sparingly soluble proteins often arise from such a near-isoelectric precipitation. Such isoelectric precipitates are usually easily dissolved by the SDS

solution used for the transfer of the IEF gel onto the SDS gel, so that the problem is limited to a loss of resolution, which, however, precludes the separation of high amounts of proteins.

The problem is, however, more severe for hydrophobic proteins when an IPG is used. In this case, a strong adsorption of the isoelectric protein to the IPG matrix seems to occur, which is not reversed by incubation of the IPG gel in the SDS solution. The result is severe quantitative losses, which seem to increase with the hydrophobicity of the protein and the amount loaded *(30)*. The sole solution to this serious problem is to increase the chaotropicity of the medium used for IEF, by using both urea and thiourea as chaotropes *(25)*. Thiourea has been shown to be a much stronger denaturant than urea itself *(31)* on a molar basis. Thiourea alone is weakly soluble in water (ca. 1 *M*), so that it cannot be used as the sole chaotrope. However, thiourea is more soluble in concentrated urea solutions *(31)*. Consequently, urea–thiourea mixtures (typically 2 *M* thiourea and 5–8 *M* urea, depending on the detergent used) exhibit a superior solubilizing power and are able to increase dramatically the solubility of membrane or nuclear proteins in IPG gels as well as protein transfer to the second-dimension SDS gel *(25)*.

The benefits of using thiourea–urea mixtures in increasing protein solubility can be transposed to conventional, carrier ampholyte-based focusing in tube gels with minor adaptations. Thiourea strongly inhibits acrylamide polymerization with the standard temed/persulfate system. However, photopolymerization with methylene blue, sodium toluene sulfinate, and diphenyl iodonium chloride *(32)* enables acrylamide polymerization in the presence of 2 *M* thiourea without any deleterious effect in the subsequent 2-D *(33)*, so that higher amounts of proteins can be loaded without loss of resolution *(33)*.

4. Concluding Remarks

Although this outline chapter has mainly dealt with the general aspects of solubilization, the main concluding remark is that there is no universal solubilization protocol. Standard urea–reducer–detergent mixtures usually achieve disruption of disulfide bonds and noncovalent interactions. Consequently, the key issues for a correct solubilization are the removal of interfering compounds, blocking of protease action, and disruption of infrequent interactions (e.g., severe ionic bonds). These problems will strongly depend on the type of sample used, the proteins of interest, and the amount to be separated, so that the optimal solubilization protocol can vary greatly from one sample to another.

However, the most frequent bottleneck for the efficient 2-D separation of as many and as much proteins as possible does not usually lie in the initial solubilization, but in keeping the solubility along the IEF step. In this field, the key feature is the disruption of hydrophobic interactions, which are responsible for

most, if not all, of the precipitation phenomena encountered during IEF. This means improving solubility during denaturing IEF will focus on the quest of ever more powerful chaotropes and detergents. In this respect, the use of thiourea may prove to be one of the keys to increase the solubility of proteins in 2D electrophoresis. One of the other keys is the use of as powerful a detergent or detergent mixtures as possible. Among a complex sample, some proteins may be well denatured and solubilized by a given detergent or chaotrope, whereas other proteins will require another detergent or chaotrope. Consequently, the future of solubilization may well be to find mixtures of detergents and chaotropes able to cope with the diversity of proteins encountered in the complex samples separated by 2-D electrophoresis.

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3

Preparation of *Escherichia coli* Samples for 2-D Gel Analysis

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1. Introduction

Escherichia coli has been studied for many years by two-dimensional polyacrylamide gel electrophoresis (2-D gels) *(1,2)*. This method has provided much information about the physiology of *E. coli*, particularly related to how the levels or synthesis rates of large numbers of proteins varied under different conditions.

In order to compare 2-D gels run in different laboratories, a number of factors need to be consistent:

1. The 2-D gel method.
2. The reference growth condition.
3. The strain.

Of these three factors, the gel method probably accounts for the largest variation because the ampholine mixtures, and immobiline strips from different manufactures give very different patterns in the first dimension. The gel pattern is very sensitive also to both the concentration of acrylamide and the pH of the Tris buffer used to make the second-dimension running gel *(3)*. Details of the 2-D gel methods can be found at the web site: http://pcsf.brcf.med.umich.edu/eco2dbase/. Other than a comparison of a B-strain with a K-12 strain *(4)*, no intense study comparing different strains has been published, but variations have been noted.

A gene-protein database of *E. coli* has been under way for over 20 yr *(2)*. This database presently includes identifications for about 400 protein spots, and information on the induction or repression of over 1600 proteins for as many as 20 experiments. There are two ways to access the data depending on

Table 1
Formulation of MOPS Buffer

Chemical	Final conc. in 1X media mM	Stock soln., g/L	Amt of stock soln. for 4 L of 10X MOPS
MOPS[a,b]	40.0	334.88/1.6	1600 mL[b]
Tricine[b]	4.0	28.64/0.16	160 mL[b]
FeSO$_4$	0.010	0.28/0.10	40 mL
NH$_4$Cl	9.52	20.32/0.20	200 mL
K$_2$SO$_4$	0.276	4.8/1.0	400 mL
K$_2$HPO$_4$	1.32	30.1/1.0	400 mL
CaCl$_2$	5×10^{-4}	0.588/0.1	0.4 mL
MgCl$_2$	0.523	10.4/0.1	40 mL
NaCl	50.0	116.88/0.4	400 mL
Micronutrients[c]			40 mL
(NH$_4$)$_6$(MO$_7$)$_{24}$	3×10^{-6}	0.0037	
H$_3$BO$_3$	4×10^{-4}	0.0247	
CoCl$_2$	3×10^{-5}	0.0071	
CuSO$_4$	1×10^{-5}	0.0025	
MnCl$_2$	8×10^{-5}	0.0158	
ZnSO$_4$	1×10^{-5}	0.0029	

[a]MOPS, potassium morpholinopropane sulfonate.
[b]Adjust pH to 7.5 using 10 M KOH; about 75 mL of 10 M KOH are required to pH both the MOPS and Tricine for 5 L of 10X MOPS.
[c]Micronutrients are made up as a single stock that contains all compounds listed. This can be stored at room temperatures for years.

whether the user is interested in obtaining data generated from some global study or running 2-D gels, and comparing gels to transfer our identifications and data to their gel system. The following section will aid the second type of user who wants to match the reference images in our database by using the same growth media, growth conditions, labeling protocols, and protein extract methods. The culture protocols in this chapter are designed for working with *E. coli*, and the growing conditions for other prokaryotes will depend on the specific organism. However, the protein extraction protocols can be used for making 2-D extracts from other Gram-negative organisms.

2. Materials

1. **Tables 1–6** give directions for making MOPS buffer (**Table 1**) *(5)*, for making supplements of amino acids (**Table 2**), bases (**Table 3**) and vitamins (**Tables 4** and **5**) *(6)*, and for formulating different growth media (**Table 6**). The composition of each medium is biologically balanced in that each nutritional component is supplied at a concentration that will support growth to approximately an optical density of 10 (420 nm).

Table 2
10X Full Supplement Amino Acids-Cysteine (10X FSA2-cys)

Amino acid, salt form	Concentration in 1X media mM	Stock soln. M	Vol. of stock soln. added for 10X FSA2^c, mL
Alanine (free)	0.8	0.8	0.5
Arginine (HCl)	0.4	0.4	0.5
Asparagine (free)	0.4	0.05	4.0
Aspartic acid (K salt)	0.4	0.4	0.5
Cysteine (HCl-H$_2$O)	0.1	0.01	—^{a}
Glutamic acid (K salt)	0.6	0.6	0.5
Glutamine (free)	0.6	0.12	2.5
Glycine (free)	0.8	0.8	0.5
Histidine (HCl-H$_2$O)	0.2	0.2	0.5
Isoleucine (free)	0.4	0.2	1.0
Leucine (free)	0.8	0.05	8.0
Lysine (HCl)	0.4	0.4	0.5
Methionine (free)	0.2	0.2	0.5
Phenylalanine (free)	0.4	0.05	4.0
Proline (free)	0.4	0.4	0.5
Serine (free)	10.0	0.4	12.5
Threonine (free)	0.4	0.4	0.5
Tryptophan (free)	0.1	0.05	1.0
Tyrosine (free)	0.2	0.01	10.0^b
Valine (free)	0.6	0.3	1.0

[a]Cysteine is not included in 10X FSA^2, and is made up at 0.01 M for use at 0.1 mM.
[b]Stock solution of tyrosine is made up in 0.01 M KOH.
[c]Total volume of 10X mix is 50 mL.

2. Diluent solution: 0.17% formaldehyde and 50 mL of 10X MOPS/500 mL. This solution is used to dilute cultures in order to read the optical density in a spectrophotometer and to stop cell growth.
3. Sonication buffer (10X): 0.1 M Trizma base, 0.05 M MgCl$_2$ · 6H$_2$O. The solution is brought to a pH of 7.4 with HCl. Dilute the 10X solution with H$_2$O prior to use *(1)*.
4. SDSBME solution: 0.3% SDS, 0.2 M DTT, 0.028 M Tris-HCl, and 0.022 M Trizma base. Aliquot (0.5 mL) the solution into microfuge tubes, and keep frozen at –70°C *(7)*.
5. DNase/RNase solution: 1 mg/mL DNase I (from Worthington), 0.25 mg/mL RNase A (Worthington), 0.024 M Tris base, 0.476 M Tris-HCl, 0.05 M MgCl$_2$. Aliquot (50 µL) into 1.5-mL microfuge tubes and store at –70°C *(7)*.
6. Lysis buffer: 9.9 M urea, 4% NP40, 0.1 M DTT, 2.2% ampholine mix. Mix together and put at 37°C to dissolve urea. Aliquot (1 mL) into microfuge tubes, and store frozen at –70°C *(1,7)*.

Table 3
10X Base Mix (Ade, Cyt, Ura, Gua)

Base	Final conc. mM	Stock soln. M^a
Adenine	0.2	0.002
Guanine	0.2	0.002
Cytosine	0.2	0.002
Uracil	0.2	0.002

^{a}Made to volume using 0.015 M KOH. The bases may be dissolved together to make a single 10X solution containing all four bases. Guanine is the least soluble and comes out of solution first. This stock solution can be stored at 4°C.

Table 4
100X Vitamin A Mix

Vitamin	Final conc., mM in 1X media	Stock soln. M^a
B1 (thiamine-HCl)	0.01	0.02
Calcium pantothenate	0.01	0.02
p-Amino benzoic acid	0.01	0.02
p-Hydroxy benzoic acid	0.01	0.02
2,3-di-hydroxy benzoic acid	0.01	0.02

^{a}Each vitamin is made as a separate stock in 0.02 M KOH, and then equal volumes are mixed together to make the mix. The 100X solution can be stored at 4°C and will turn brown.

7. 5% TCA: Add to this solution the chemical form of the isotope being used. If the radioisotope is ^{35}S-Met, add 0.02 M Met.

3. Methods

The medium used for growing cells for all of the reference images in the *E. coli* gene–protein database is MOPS medium supplemented with glucose and any needed vitamins (*see* **Note 1**). For Figs. 2 and 3 in **ref. 2**, protein extracts were prepared by sonication. For Figs. 1 and 4 in **ref. 2**, protein extracts were prepared by the SDS method. The following sections give methods for growing cultures, radiolabeling cultures, and preparing protein extracts.

3.1. Methods for Growing Cultures

3.1.1. Overnight Cultures

1. Grow overnight cultures at the temperature the culture will be grown the following day (*see* **Note 2**). In most cases, a liquid culture should be started from a single colony on a plate preferably of the same composition as the liquid media to be used (*see* **Note 3**).
2. The overnight culture should reach an OD$_{420\ nm}$ of about 1.0 (3×10^8 cells/mL).

Table 5
50X Vitamin B Mixture

Vitamin	Final conc. m M in 1X media	Stock soln. M^a
Riboflavin	0.01	0.001
Biotin	0.01	0.002
Nicotinic acid (niacin)	0.01	0.01
Pyridoxine-HCl	0.01	0.01

[a]Each vitamin is made up in water, and then can be added together to make the mix using 1.0 mL of stock riboflavin, 0.5 mL of biotin, 0.1 mL of nicotinic acid, 0.1 mL of pyridoxine-HCl, 0.3 mL of H_2O.

Table 6
Recipes for Different Growth Media

Stock solution	Glu limiting[a]	Glu min[a]	Glu Rich[a]
35.7% Glucose	0.1 mL	1.0 mL	1.0 mL
10X MOPS	10.0 mL	10.0 mL	10.0 mL
10X FSA^2-cys[b]			10.0 mL
100X cysteine[b]			1.0 mL
10X Ade, cyt, ura, gua[b]			10.0 mL
100X Vitamin mix A[b]			1.0 mL

[a]Per 100 mL total.
[b]*See* description of 10X Ade, Cyt, Ura, Gua; 10X FSA2-cys; 100X cysteine, 100X vitamin supplements the previous tables.

3.1.2. Monitoring the Growth of Liquid Cultures (Steady-State Growth)

1. Dilute the overnight culture at least 1/20 (to OD 0.05) and if possible 1/100 (to OD of 0.01) with fresh media.
2. Monitor the growth of a liquid culture with minimum disturbance of growth. Small samples are removed by stopping the shaker bath for the briefest time required to obtain the sample.
3. For the spectrophotometer accepting 1-cm path cuvets, a 0.5-mL sample of the culture is mixed with 2.0 mL of diluent solution. Optical density is measured at 420 nm in most cases. An $OD_{420\ nm}$ of 1.0 using a 1-cm light path corresponds to about 3×10^8 cells/mL or 0.1 mg of protein. This is approximately equal to $OD_{600\ nm}$ of 0.5 (*see* **Note 4**).
4. Put cultures on ice water and chill immediately.
5. Transfer the culture to a microfuge tube and spin at 12,000g (10,000 rpm in a microfuge) for 10 min at 4°C.
6. Remove the supernatant, and either freeze the samples at –70°C or make the protein extracts for 2-D gels.

3.2. Radiolabeling Cultures

Usually only a small portion (1 mL) of the culture is radiolabeled. It is important to keep the culture conditions constant while labeling. Scintillation vials (20 mL) with foam caps work well for labeling 1-mL portions (*see* **Note 5**). Regardless of whether protein, lipids, DNA, or RNA is being radiolabeled, the labeling is usually done under either steady-state conditions (net accumulation of product over time) or pulse–chase conditions (synthesis rates and degradation rates of products). Pulse–chase labeling is done to determine if the rate of synthesis of individual proteins is changing as a result of some change in the growth condition of the culture.

3.2.1. Steady-State Labeling

1. Dilute an overnight culture, and add isotopic label to the growing culture at an OD of 0.1. For steady-state labeling of ^{35}S-methionine, one-tenth the normal methionine concentration (*see* **Table 2**) is added to the media with 0.01 mL of ^{35}S-methionine (1000 Ci/mmo, 10 mCi/mL) for a final SA of 0.5 mCi/mmol.
2. Harvest the culture at $OD_{420\ nm}$ of 0.8 (0.2 OD units before the methionine is exhausted from the media) (*see* **Note 5**).
3. Put cultures on ice water, and chill immediately after the labeling.
4. Transfer the culture to a microfuge tube, and spin at 12,000g (10,000 rpm in a microfuge) for 10 min at 4°C.
5. Remove the "hot" supernatant. Using Q-tips, remove excess liquid from the pellet after decanting.
6. Freeze samples at –70°C or make protein extracts for 2-D gels.

3.2.2. Pulse-Chase Labeling (*see* **Note 7**)

1. Dilute an overnight culture with fresh media, and grow until a steady-state condition is reached (*see* **Note 4**).
2. Prior to initiating the change in the culture conditions, determine the OD at the start and end of the pulse, and calculate the amount of the chemical form of the isotope that will be needed (*see* **Note 8**).
3. Add the isotopic label at the start of the change in the culture condition.
4. At the end of the labeling period, add 100- to 500-fold excess of the nonradioactive chemical form of the isotope. If ^{35}S-Met is used for the label, 0.167 mL of a 0.2 *M* Met chase solution should be used for each 1 mL of culture (*see* **Note 9**).
5. Put cultures on ice water, and chill immediately after the labeling and chase period.
6. Transfer the culture to a microfuge tube, and spin at 12,000g (10,000 rpm in a microfuge) for 10 min at 4°C.
7. Remove the hot supernatant, and wash the pelleted cells with cold medium.
8. Use Q-tips to remove excess liquid from the pellet after decanting.
9. Freeze samples at –70°C, or make protein extracts for 2-D gels.

3.3. Sonicated Protein Extracts (see Note 10)

1. For sonicated extracts, resuspend the cell pellet in cold sonication buffer. For a 10-mL culture (pelleted in a microfuge tube), the cell pellet is resuspended in 0.1 mL of sonication buffer.
2. Using a microtip for the sonicator, place the tube over the tip without touching the sides or bottom of the tube. Place ice water in a large test tube over the microfuge tube to cool the sample (*see* **Note 11**). Wrap a small tissue over the top of the microfuge tube to prevent the liquid from spraying out.
3. Sonicate the cells with 3–4 × 5-s bursts using the lowest power setting.
4. Add DNase/RNase solution (1 μL for every 50 μL of extract), and leave the mixture on ice for 10 min.
5. Add urea (1 mg/μL of extract), followed by equal volume of lysis buffer. For example, if the cell pellet had been resuspended in 0.1 mL, 100 mg of urea, and 0.1 mL of lysis buffer is added.

3.4. SDS Protein Extracts (see Note 10)

1. For SDS extracts, resuspend the cell pellet in SDSBME solution such that the protein concentration in the SDSBME solution is 1–5 μg/mL.
2. Place the extracts in a boiling water bath for 2 min, and then cool on ice.
3. Add DNase/RNase solution ($\frac{1}{10}$ the volume of SDSBME), and leave the mixture on ice for 10 min.
4. Add lysis buffer (four times the volume of SDSBME solution). The typical extract from 1 mL of culture at $OD_{420\,nm}$ of 0.5 would use the following volumes of each solution: 30 μL of SDSBME, 3 μL of DNase/RNase, and 120 μL of lysis buffer.

3.5. Measuring Specific Activity

1. Add 3 μL of the protein extract to a microfuge tube containing 0.5 mL of 5% TCA.
2. Leave the mixture on ice for 30 min.
3. Collect the TCA precipitate on a glass fiber filter; wash the filtering apparatus with water between samples.
4. Place the glass fiber filter in a scintillation vial with appropriate scintillation cocktail for aqueous samples and count.

4. Notes

1. The 10X MOPS buffer can be made without phosphate, sulfate, or ammonia if alternate sources of these nutrients are used or if radiolabeling requires reduced quantities of these nutrients. This buffer can be stored for long periods of time at –20°C. The guanine in the 10X Ade, Cyt, Ura, Gua supplement does not stay in solution very long. The guanine can be made as a separate supplement.
2. The preferred medium for overnight cultures is a glucose-limiting MOPS. *E. coli* seems to survive starvation for carbon very well and also recovers from this stationary state more rapidly than the stationary state reached at high cell densities. If a culture is to be grown in glucose minimal media, then the strain should be

started on a glucose minimal plate. When working with strains containing a plasmid, minicell-producing strains, which will be used for producing minicells, or colicin-producing strains, which will be used to produce colicin, a loopful of cells from plates should be used to start the liquid culture.

3. Liquid cultures are always grown in a glass flask that is 5–10 times larger than the volume of culture (e.g., for a 10-mL culture, use a 100-mL Delong flask with a stainless-steel top). Glass flasks allow for fast and even heat exchange, and the excess flask volume allows proper mixing and aeration for aerobic and anaerobic cultures. Anaerobic cultures should be grown in an anaerobic bag where heat maintenance and aeration can be achieved using a double-walled beaker through which water circulates (water temperature is controlled by a circulating water bath) *(8)*. The culture flask containing a stir bar is inserted into the beaker and covered with sand. The sand allows for an even heat distribution. Aeration is maintained by placing the beaker on a magnetic stir plate.

4. Steady-state growth is usually achieved when a plot of time vs the log of OD yields a straight line for three to five doublings. Most wild-type *E. coli* strains reach steady-state growth very rapidly, especially when the overnight culture is glucose-limiting MOPS. If steady-state growth is not reached by $OD_{420 \, nm} = 1$, then it is necessary to dilute the culture 1/10 with fresh, prewarmed media, and to continue monitoring growth. Above an $OD_{420 \, nm}$ of 2.0, the culture must be considered to be in the transition to stationary phase.

5. Labeling is not advised in plastic containers or test tubes. We have found that 20-mL scintillation vials with foam caps work well for 1-mL labeling.

6. For steady-state labeling, the key factor is that the label is continually incorporated over several generations. In most cases, the specific activity of the radiolabel must be decreased from the stock solution. For example, the specific activity of ^{35}S-methionine from most suppliers is usually about 1000 Ci/mmol, and the concentration about 10 mCi/mL. At this high specific activity and concentration, labeling at 100 μCi/mL supplies only 10^{-5} mM of methionine. Using **Table 2**, that is enough methionine for 0.005 OD units (at 420 nm) of culture; thus, nonradioactive methionine needs to be added.

7. To compare the level or synthesis rate of individual proteins in different conditions, a double-labeling protocol is extremely useful. A reference culture is radiolabeled with one isotope and mixed with each experimental culture, labeled with a different isotope. The reference radiolabel is used as the baseline for comparing different experimental cultures. Typically, steady-state labeling with a ^{3}H-amino acid is used for the reference labeling. The experimental condition is radiolabeled with a ^{14}C or ^{35}S-amino acid either in steady state or pulse chase. Equal volumes of the reference culture are mixed with each experimental culture prior to harvesting the cells.

8. For example, 5 μL of ^{35}S-met (1000 Ci/mmol; 10 mCi/mL) have about 0.006 μg of Met. A 5-min pulse of a culture at an OD420 nm = 0.3 and growing at a doubling time of 60 min will require 0.005 μg of Met (per 1 mL of culture). Base the calculation on the following estimations—*E. coli* needs 0.2 mM or 30 μg/mL

of Met to grow to an $OD_{420 \, nm}$ of 10. Labeling of this sort may require some trial and error to obtain the appropriate incorporation.

9. The chase should be a consistent time and sufficient to allow all of the peptides initiated at the end of the pulse to be completely translated. (At 37°C it takes 3 min to translate a large protein, such as β-galactosidase.)

10. Sonicated extracts will contain soluble proteins and some peripheral membrane proteins, but integral membrane proteins (i.e., outer membrane porins) will not be solubilized by this method. SDS extraction will sufficiently solubilize outer membrane proteins to allow separation on 2-D gels.

11. The lysis buffer used for making protein extracts contains urea and should not be heated beyond 37°C. On heating, urea breaks down, and the resulting modifications cause carbamylation of proteins, resulting in streaking in the isoelectric focusing dimension.

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4

Preparing 2-D Protein Extracts from Yeast

David M. Schieltz

1. Introduction

The yeast *Saccharomyces cerevisiae* became the first eukaryotic organism to have its entire genome sequenced *(1)*. With the completion of the genome, over 6000 genes on 16 chromosomes were identified. Several laboratories are undertaking the task of identifying the yeast proteins on two-dimensional (2-D) gels with the goal of developing a yeast protein database for studying global changes in protein synthesis, protein modification, and protein degradation that result from environmental or genetic changes *(2,3)*. This chapter provides protocols for labeling yeast with ^{35}S-methionine and the preparation of 2-D extract using methods developed by Garrels et al. and Boucherie et al. *(2,3)*. Additional protocols for preparing yeast 2-D extracts can be found at the Geneva University Hospital's Electrophoresis Laboratory, which can be accessed via the World Wide Web at http://expasy.hcuge.ch/ch2d/technical-info.html and at the University of Göteberg's Lundberg Laboratory, which can be accessed at http://yeast-2dpage.gmm.gu.se/sacch/immobiline/methods/labelling.

2. Materials
2.1. Equipment

1. Mini-Beadbeater (Biospec Products, Bartlesville, OK).
2. 0.5-mm diameter glass or zirconia beads (Biospec Products, Bartlesville, OK).
3. Speed Vac.

2.2. Reagents

1. SD media: 0.67% Bacto-yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 2% glucose, pH 5.8. Mix 6.7 g of yeast nitrogen base without amino acids in a volume of 800 mL of dH$_2$O, and adjust the pH to 5.8 with 10 *N* NaOH. Adjust the final volume to 950 mL with dH$_2$O and autoclave. After auto-

claving, add 50 mL of 40% glucose solution. Depending on the growth requirements of the yeast strain, supplement the media with the appropriate amino acids or supplements to a final concentration of 100 μg/mL.

2. 40% Glucose solution: 40% (w/v) glucose. Dissolve 200 g of glucose in a total volume of 500 mL of dH$_2$O and filter-sterilize.
3. 2X SDS solution: 0.6% SDS, 2% β-mercaptoethanol, 0.1 *M* Tris-HCl, pH 8.0.
4. Lysis buffer: 20 m*M* Tris-HCl, pH 7.6, 10 m*M* NaF, 10 m*M* NaCl, 0.5 m*M* 0.1% deoxycholate. Add the protease inhibitor cocktail to the lysis buffer just before use.
5. Protease Inhibitor cocktail (1000X): 1 mg/mL leupeptin, 1 mg/mL pepstatin, 10 mg/mL *N*-tosyl-L-phenylalanine chloramethyl ketone (TPCK), 10 mg/mL soybean trypsin inhibitor, 1 *M* 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Add to the lysis buffer just prior to use.
6. DNase/RNase/Mg mix: 0.5 mg/mL DNase I, 0.25 mg/mL RNase A, 50 m*M* MgCl. The purity of the DNase I and RNase A is critical, since exogenous proteases create serious problems. DNase I and RNase A from Worthington have proven to be satisfactory.
7. 1-D buffer: 9 *M* urea, 4% CHAPS, 2 m*M* DTT, 2% 6–8 ampholytes.
8. YPD media: 2% peptone, 1% yeast extract, 2% glucose.

3. Methods

3.1. Growing and Labeling Yeast

The protocols for growing and labeling yeast are adapted from Garrels et al. and Boucherie et al. *(2,3)*.

1. Grow cultures of yeast in SD media with the appropriate amino acids overnight at 30°C. Typically, a 50-mL culture is grown in a 250-mL flask with rotary shaking at 30°C.
2. Using the overnight culture, inoculate 50 mL of fresh SD media in a 500-mL flask to a cell density of approx 5×10^4 cells/mL.
3. Grow the cells with rotary shaking at 30°C until the cells reach an OD$_{600}$ of 1 (*see* **Note 1**).
4. Transfer 1 mL of the culture to a test tube, add 100 μCi of ^{35}S-methionine (>1000 Ci/mmol), and label for 30 min (*see* **Note 2**).
5. Proceed immediately to the cell lysis protocol.

3.2. Cell Lysis Protocol

All the steps below should be rapidly carried out at 4°C to avoid unwanted proteolytic activity.

1. Transfer the cells to a 1.5-mL microcentrifuge tube, and pellet the cells at 5000*g* for 2 min (*see* **Note 3**).
2. Add the protease inhibitor cocktail to the lysis buffer.
3. Remove the supernatant, and resuspend the cells in 100 μL of lysis buffer.
4. Transfer the resuspended cells to a 1.5-mL screw-cap microcentrifuge tube containing 0.28 g of glass beads (0.5 mm), and vortex vigorously for 2 min (*see* **Note 4**).
5. Centrifuge the sample at 5000*g* for 10 s.

6. Withdraw the liquid from the beads using a fine pipet tip, and transfer to a prechilled 1.5-mL screw-cap microcentrifuge tube containing 7 µL of the DNase/RNase/Mg mix.

7. Incubate the mixture on ice for 2 min.

8. Add 75 µL of the 2X SDS solution, and immediately plunge into boiling water for 1 min.

9. Plunge the tube into ice and cool.

10. Centrifuge the tube at 14,000g for 30 s (*see* **Note 5**).

11. Transfer the supernatant to a fresh 1.5-mL screw-cap microcentrifuge tube, and freeze the sample on liquid nitrogen (*see* **Note 6**).

12. Lyophilize the sample in a Speed Vac for 2 h.

13. Resuspend the sample in one-dimensional gel electrophoresis buffer (1-D buffer) buffer in a volume that is equal to the solution prior to lyophilization. Store at −80°C or immediately load onto the 1-D gel buffer.

14. Typically, 4–10 µL of the extract are loaded onto each 2-D gel (*see* **Note 7**).

4. Notes

1. An OD$_{600}$ of 1 corresponds to a midlog phase culture with a cell density of approx 1–2 × 10^7 cells/mL.

2. This protocol is based on labeling with ^{35}S-methionine, but any ^{14}C-, ^{32}P-, ^{35}S-radio-isotopes precursor can be used. For yeast grown in media without methionine, exogenous methionine is transported into and used by the cell for protein synthesis under many metabolic conditions. The protocol can be modified to account for different media, incubation times, and labeled precursors.

3. After pelleting, the cells can be washed twice with ice-cold water, and the cell pellet stored at −70°C.

4. An alternative to vortexing by hand is the use of the Mini-Beadbeater (Biospec Products, Bartlesville, OK). The tube is inserted into the Mini-Beadbeater and shaken at high speed for 1 min at 4°C. Zirconia beads (0.5 mm, Biospec Products) can be used in place of the glass beads and reportedly improve cell disruption.

5. To assay the radioactivity, remove 2 µL for TCA precipitation (*see* Chapter 13). To assay protein concentration, remove 4 µL for the Bradford assay (*see* Chapter 12). It is usually satisfactory to estimate protein concentration from an unlabeled pilot experiment.

6. The sample can be stored at −80°C at this step.

7. Typically, 500,000 dpm with 10 µg or less of protein is loaded onto the 2-D gel.

Acknowledgments

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5

2-D Protein Extracts from *Drosophila melanogaster*

Christer Ericsson

1. Introduction

Although the majority of proteins expressed in organisms with relatively low protein complexity, such as *Escherichia coli*, can be resolved and detected in a single gel *(1,2)*, this is not true for more complex organisms, such as *Drosophila melanogaster*. A smaller fraction of the total complement of proteins can be detected in a single gel as the protein complexity of an organism increases. With current detection techniques, this situation can be improved by analyzing subfractions separately and then matching them to each other, preferably using computer-assisted, gel-matching techniques. It has been estimated that about 8% of the proteins encoded by the genome can be analyzed in a single 2-D PAGE of a total protein extract of *D. melanogaster (3)*. By matching subfractions, that value can be increased dramatically.

Reference protein patterns and the increasing number of proteins identified on 2-D PAGE are conveniently maintained as image-based databases accessible as image maps in the World Wide Web format (WWW). A *D. melanogaster* 2-DE Protein Database is available at http://tyr.cmb.ki.se *(4)*. Those protein spots that have been identified are highlighted and clickable. HyperText links are provided to the SWISS-PROT (http://espasy.hcuge.ch) database of protein sequences and the FlyBase (http://flybase.harvard.edu) database of genetic information. Comparison of an experimental gel pattern with such a reference gel can provide immediate information on protein identities, and on differences and similarities in the protein patterns between the two samples.

Our technique for preparing adult tissue total protein and brain membrane proteins, by extraction of homogenized frozen tissue is given below. The pioneering development of freeze-drying and subsequent dissection as a method

to obtain individual tissues *(5)*, and the fresh preparation of in vitro radioactively labeled imaginal tissues from larvae should also be recalled *(7)*. The former method has been shown to be compatible with, and complementary to, our techniques of sample preparation *(4)*. The choice of sample preparation method should be based on the requirements of the particular investigation.

The most sensitive technique for detecting proteins in the rapidly differentiating tissues of embryos and the rapidly growing imaginal tissues of larvae is radioactive metabolic labeling. Silver staining *(6)* is the most sensitive staining technique for the slowly turning over proteins in adult tissues.

2. Materials
2.1. Equipment

1. Brass sieves to separate fly parts: A top sieve of Tyler equivalent 16 mesh (1-mm opening) will retain bodies, but not heads, wings, legs, or antennae. A center sieve of Tyler equivalent 32 mesh (0.5-mm opening) will retain heads, but not wings, legs, or antennae. A bottom pan will collect wings, legs, and antennae. Sieves can be obtained from, for example, Fisher (Hampton, NM).
2. Mortar and pestle made from agate, or other material, that does not crack during repeated freeze–thaw cycles for grinding up samples.

2.2. Solutions and Reagents

1. Immobiline® sample solubilization solution: 1% (w/v) sodium dodecyl sulfate (SDS) solution, 5% (v/v) β-mercaptoethanol. Store aliquoted at –20°C.
2. Immobiline sample dilution buffer: urea 13.5 g β-mercaptoethanol 0.5 mL or DL-dithiothreitol (DTT) 250 mg, Pharmalyte 3-10 ampholytes (Pharmacia Biotech, Uppsala, Sweden) 500 μL, Triton X-100 130 μL, a few grains of bromphenol blue (BPB). Make up to 25 mL. Store aliquoted. Can be stored at –20°C for 2 mo.
3. Immobiline DryStrip, pH 3.5–10, NL, 18-cm (Pharmacia Biotech), immobilized pH-gradient isoelectric focusing gels for the first-dimension separation step.

3. Methods
3.1. Protein Sample Preparation

1. Grow adult flies either on conventional complex medium or on a defined amino acid medium *(8)*. If any food protein, present in the gut, is suspected of obscuring the protein pattern of interest, the defined medium should be chosen. The flies can be aged by transferring newly eclosed flies to a new vial for a given time.
2. Harvest flies by shaking the living flies into a 50-mL screw-capped plastic tube held in liquid nitrogen and fitted with a plastic funnel to ease the transfer. When exposed to the cold, the flies immediately freeze and can subsequently be stored at –70°C or below (*see* **Note 1**). In our procedure, it is important that the tissues never thaw between collection and solubilization in electrophoresis buffer in order to avoid proteolysis. If the flies are to be sorted prior to electrophoresis,

this should be done either prior to freezing or, if after freezing, on a microscope equipped with a cold stage that can be set to subzero temperatures (*see* **Note 2**).

3. Collect sorted flies in 50-mL screw-capped plastic tubes on liquid nitrogen.
4. Separate the heads from the bodies, wings, legs, and antennae by vigorous shaking in the cold followed by sieving through a stack of sieves precooled to –70°C. Mass isolation of heads is conveniently performed by first braking them off at the neck by vigorously shaking frozen flies in a 50-mL screw-capped tube at –70°C (*see* **Note 3**). Brass sieves, available in a variety of meshes, are useful for separating the body parts. As an initial guide, Tyler equivalent 16 mesh can be used to isolate the bodies, and Tyler equivalent 32 mesh can be used to isolate the heads. Wings and legs are collected in the bottom. These measures should be regarded as guidelines only, since the sizes of different strains of flies vary to some extent. Heads from *eya* flies are smaller than wild-type, for example. It is important that the sieves be prechilled to –70°C, and remain frozen throughout this procedure or ice will form, reducing the effective mesh size and not separating the body parts as intended (*see* **Note 4**). To separate the parts, they are allowed to roll over the surface of the sieve. We routinely repeat this six times to achieve efficient separation.
5. Collect the body parts accumulated on the various sieve surfaces in the cold, into separate tubes and keep frozen at –70°C (*see* **Note 5**).
6. Recover the clean, isolated bodies, consisting of thoraxes and abdomens, but no wings or legs on the upper sieve; recover clean isolated heads on the lower sieve, and legs and wings in the bottom pan. A clean separation and quantitative recovery should be verified at this stage using a dissection microscope.
7. The bodies can either be extracted as is, or further dissected, on a cold stage, into separate thoraxes and abdomens, using a scalpel cooled by dipping periodically into liquid nitrogen. It is convenient to use a pair of thin cotton gloves during these procedures to avoid transferring heat to the samples.

3.2. Total Protein Extraction

Having obtained the clean samples, it is very important to mince them effectively. Failure to do so will result in selective extraction of protein, which will distort the results of the experiment (*see* **Note 6**).

1. Suspend about 100 µL of body parts in an equal volume of 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol in a 1.5-mL Eppendorf tube (*see* **Note 7**).
2. Rapidly freeze the tubes by placing the tube in a metal rack (from a heat block) immersed in liquid nitrogen.
3. Loosen the frozen plug of tissue within the tube by pounding the tube, top down, onto the lab bench.
4. Transfer the frozen plug to a prechilled agate mortar at –70°C on a metal block standing in liquid nitrogen. The tissue should be ground in the mortar until it becomes a homogenous white powder, and no tissue fragments and no brown cuticular coloring are seen (*see* **Note 8**).
5. Carefully scrape the finely ground tissue into an Eppendorf tube for subsequent solubilization. *See* **Subheading 3.4.**

3.3. Membrane Protein Preparation

Membrane proteins can be isolated from isolated heads by homogenization and differential centrifugation essentially as described previously *(9)*.

1. Suspend 1 g of isolated *D. melanogaster* heads (~2 mL), prepared as described in **Subheading 3.1.**, in a minimum volume of homogenization buffer, and freeze on liquid nitrogen.
2. Grind the heads into a fine powder in a prechilled (at –70°C) agate mortar. The mortar is kept cold by placing it on a heat block in liquid nitrogen.
3. Thaw the homogenate, and dilute to 10 mL in homogenization buffer.
4. Homogenized by hand by 10 strokes in a 17-mL, all-glass, Kontes Potter-Elvehjem homogenizer at 4°C in a cold room, on ice.
5. Centrifuge the homogenate at 1000*g* for 10 min at 4°C in a prechilled 50-mL polycarbonate tubes.
6. Transfer the supernatant into prechilled 5-mL polyallomer tubes, and centrifuge at 105,000*g*, at 4°C, for 30 min.
7. Dissolve the pellet that constitutes the membrane protein fraction in solubilization buffer as described in **Subheading 3.4.** The membrane protein fraction was estimated by quantitative Western blot analysis to be approximately fivefold enriched in the membrane proteins compared to a total protein extract. It contains more than 90% of the *N*-glycosylated proteins. About two-thirds of the membrane proteins were estimated to be *N*-glycosylated *(10)*.

3.4. Protein Solubilization

1. Solubilize the proteins by heating to 95°C for 5 min in the Immobiline sample solubilization solution and vortexing. The SDS helps to solubilize the proteins. This step may be especially important for efficient solubilization of membrane proteins.
2. Sediment the fragmented cuticle remnants (if present) in a tabletop centrifuge at 8000*g* for 5 min. The SDS does not interfere with the subsequent isoelectric focusing (IEF) if diluted to <0.25% (at least fourfold) with Immobiline sample dilution buffer, prior to electrophoresis. A similar procedure can be used for conventional IEF gels or conventional nonequilibrium pH-gradient electrophoresis (NEpHGE) first-dimension 2-DE experiments *(3)*.
3. The amount of protein extracted can be estimated by TCA precipitation onto nitrocellulose membranes, followed by amido black staining, using bovine serum albumin (BSA) as the standard *(11)*.
4. Just prior to electrophoresis, centrifuge the sample for 15 min in a tabletop centrifuge at 8000*g* to eliminate any insoluble material (*see* **Note 9**).

3.5. Immobilized pH Gradient First Dimension

For the first-dimension separation, where the proteins are separated by charge, we use a wide-range immobilized pH-gradient gel, with pH values ranging from 3.5 to 10. This gel allows us to separate most proteins in a single gel. Traditional IEF only separates in the acidic to neutral range. For basic

proteins, a separate NEpHGE first-dimension gel is needed. Another advantage of the immobilized pH gradient is that it makes the experiments very reproducible, since the pH gradient is immobilized by covalent crosslinking to the gel matrix, under tight quality control. These gels can be purchased premade from Pharmacia under the trade name Immobiline. About 50 μg of either total extractable protein or membrane protein extracts can be resolved by IEF on Immobiline® DryStrip, pH 3.5–10.0, NL, 18-cm (Pharmacia Biotech) first-dimension gels. In the second-dimension separation, where the proteins are separated by size, we use traditional SDS-PAGE. The composition of this gel should be selected to resolve proteins in the mol-wt range of interest *(12)*.

4. Notes

1. An important alternative way of collecting adult tissues based on freeze-substitution was developed by Hotta and collegues *(5)*. This procedure is especially amenable to clean isolation of individual tissues, by dissection, with no detectable proteolysis. Although more tedious than the present method of sample collection and not amenable to mass isolation to the same extent, it provides unsurpassed ability to isolate individual tissues. We have determined that tissue extracts prepared by this alternative method are compatible with our procedures.

2. It should be verified that the stage actually reaches freezing by continually monitoring the temperature and the appearance of frost. The fly bodies should all be in contact with the stage in order to stay frozen. No piling of flies or standing on their legs must be allowed, since the upper layers thaw instantly.

3. We routinely pound the tube into the wall of a –70°C freezer 100 times, wearing cotton gloves to minimize heat transfer. Care must be taken so the tube does not break during this procedure, or all fly material could be lost on the bottom of the freezer. Alternatively, the tube of frozen flies can be taken to a cold room, on dry ice, and pounded rapidly on a rubber pad. Care must be taken to maintain the flies frozen at all times. The yield of severed heads is in excess of 80%.

4. If the sieves ice over, they should be thawed, washed, carefully dried, and then precooled at –70°C again, before attempting any further separations. If the fly homogenate is allowed to thaw at this stage it will stick to the sieves.

5. The fragmented legs and wings tend to form a powder that clings to the other body parts if not removed at an early stage. We remove the fragmented legs and wings by "panning" the homogenate three times, or as required until clean, on a Whatman 3MM filter paper at –70°C. The legs and wings stick to the filter paper while the body parts roll off. The parts are then poured onto the top sieve, which is precooled at –70°C. After the separation is complete, use a large funnel to facilitate transfer of the separated parts from the sieves to the tubes.

6. Directly grinding of frozen tissues in liquid nitrogen does not result in a sufficiently fine mincing for efficient extraction. In our experience, the tissue must be frozen first into a block of ice, which is then ground, using a mortar and pestle, on liquid nitrogen. The ice helps to break up the tissue very efficiently.

7. Because of the waxy cuticle and airways inside the heads, they tend to float on top of the buffer. They can be quickly and completely immersed in the buffer by gently tapping the tube on the benchtop.

8. It is important to use a mortar that does not crack at the low temperatures used. We use a small agate mortar standing on a metal block immersed in liquid nitrogen. Resting it on the metal block cools the pestle. Both mortar and pestle should be precooled by freezing for several hours at –70°C. It is helpful to create a collar of aluminum foil around and above the mortar to prevent loss of frozen tissue chips during the homogenization.

9. Sample precipitates at the application point are perhaps the most common single cause of experimental failure. There should be no visible precipitate at this point. If there is, the cause should be determined and eliminated. Simply spinning out a large precipitate is likely to result in selective solubilization that will distort the experiment.

Acknowledgments

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6

Preparing 2-D Protein Extracts
from *Caenorhabditis elegans*

Robert Zwilling

1. Introduction

The small nematode *Caenorhabditis elegans* (*C. elegans*, 1 mm in length) originally was introduced into the laboratory in 1965 by Brenner *(1)* and has since become an important model organism. *Caenorhabditis elegans* for a variety of reasons offers excellent conditions for the study of basic features of life *(2)*. *C. elegans* is surrounded by a rigid cuticula, which is, however, completely transparent. The adult organism is composed of a constant number of somatic cells (959 in the adult hermaphrodite, 1031 in the adult male), of which the complete cell lineage during development is known in all details *(3)*. Altogether, this makes *C. elegans* a very suitable model organism to approach fundamental problems in development and aging.

A full understanding of the dynamics of gene transcription and protein expression in this organism will be possible only if—in addition to the genetic studies—we are also able to decipher the totality of the translational products and their posttranslational modifications. We, therefore, have created as a basis for the detailed study of protein expression in *C. elegans* a 2-D PAGE map, which resolves up to 2000 spots/gel within the window of p*I* 3.5–9 and M_r 10–200 kDa *(4)* (**Fig. 1**). This method will also permit the investigation of stage-specific protein expression in *C. elegans*. Therefore, under **Subheadings 3.4.** and **3.5.**, the preparation of eggs (*see* **Notes 7** and **8**) and of larval stages L_1–L_4 is also described.

It is possible to breed *C. elegans* in liquid medium or alternatively on agar plates. The first method gives access to relatively large quantities of proteins and, therefore, is described in this protocol. The agar plate culture technique is easier to perform and is suited to maintaining *C. elegans* reference strains over
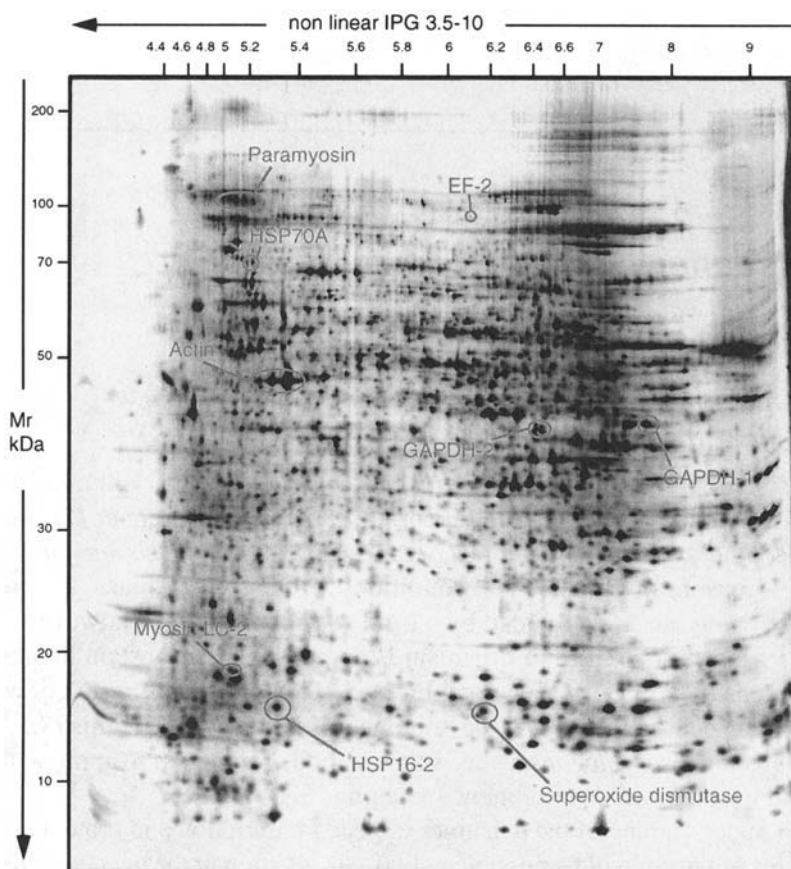
Fig. 1. 2-D gel electrophoresis of a *C. elegans* protein extract. Spots were assigned tentatively on the basis of the apparent p*I* and *M*$_r$. The image resulted from a cooperation between L. Bini, V. Pallini (both Siena), and R. Zwilling (Heidelberg) *(4)*.

prolonged periods (*see* **Note 1**). For further information on basic culture methods of *C. elegans*, *see also* Epstein and Shakes *(5)*.

2. Materials

2.1. Animals and Reagents

1. The *C. elegans* wild-type strain N2 variant Bristol and the *Escherichia coli* strain OP50 are available from the *Caenorhabditis* Genetic Center, University of Minnesota, St. Paul, MN.
2. M9 washing buffer: 44 m*M* KH$_2$PO$_4$ (6 g/L), 21 m*M* Na$_2$HPO$_4$ · 2H$_2$O (3.8 g/L), 86 m*M* NaCl (5 g/L) adjust to pH 7.0 and autoclave. After autoclaving, add 1 mL/L of 1 *M* stock solution of MgSO$_4$ for a final concentration of 1 m*M*.

3. Sucrose solution: 70% saccharose (350 g/0.5 L) dissolved in sterile Milli-Q water.
4. Freezing solution: 5.6 g/L NaCl, 6.8 g/L KH$_2$PO$_4$, 300 g/L glycerine.
5. Dissolution medium: For 10 mL solution, dissolve 5.4 g urea, 0.4 g CHAPS detergent (Sigma C3023), 0.1 g DTE, 0.0424 g Tris in ddH$_2$O. Store at –20°C in small aliquots.
6. Alkaline hypochloride solution: Mix 15 mL 12.5% NaOCl with 135 mL of 0.1 M NaOH for a total volume of 150 mL.

2.2. Nutrient Solution Recipes

1. S medium (*C. elegans* liquid culture): Combine 1000 mL S-basal, 10 mL trace metal solution, 10 mL of 1 M, pH 6.0, K-citrate, 3 mL of 1 M CaCl$_2$, and 3 mL of 1 M MgSO$_4$. All components are autoclaved separately for 20 min and then combined.
2. S basal (Component of S medium): 5.8 g NaCl, 5.9 g KH$_2$PO$_4$, and 1.2 g K$_2$HPO$_4$ (*see* **Note 5**) are dissolved in ddH$_2$O to a total volume of 1000 mL, adjusted to pH 6.0, and autoclaved for 20 min. After autoclaving, add 1 mL of cholesterol (5 mg/mL ethanol).
3. Trace metals solution (component of S medium): 5 mM Na$_2$EDTA (1.86 g/L), 2.5 mM FeSO$_4$ · 7H$_2$O (0.69 g/L), 1.0 mM MnCl$_2$ · 4H$_2$O (0.2 g/L), 1.0 mM ZnSO$_4$ · 7H$_2$O (2.29 g/L), 0.1 mM CuSO$_4$ · 5H$_2$O (0.025 g/L). Dissolve in ddH$_2$O, autoclave for 20 min, and store at 4°C in the dark.
4. Standard I medium (for growing *E. coli*): peptone (15.0 g/L), yeast extract (3 g/L), NaCl (6 g/L), D(+)-glucose (1 g/L). Dissolve in ddH$_2$O and autoclave.

3. Methods

3.1. Preparation of a E. coli culture

The nutrition of *C. elegans* is based on bacteria. We chose for this purpose the *E. coli* strain OP 50 (*see* **Note 2**).

1. Eight 1-L Erlenmeyer flasks containing 250 mL standard I medium each are autoclaved for 20 min.
2. After cooling down, inoculate each flask under sterile conditions with 100 µL *E. coli* suspension.
3. Incubate at 37°C overnight in a shaker.
4. Before centrifugation in the following step 1-mL aliquots are taken from the culture medium and stored at 4°C as stock suspension.
5. Harvest the bacteria by centrifugation at 5000g for 10 min.
6. Store the pellet at –20°C until use.

3.2. Growing of C. elegans in Liquid Medium

1. Sterilize four Fernbach flasks by autoclaving (*see* **Note 3**).
2. Add 250 mL of nutrient medium S into each flask containing 5–10 g packed *E. coli* cells.
3. The flasks are then inoculated with 200 µL of sedimented *C. elegans* liquid suspension each.

4. Compressed air is dispensed through a valve and two sterile filters (0.22 μm), and via a thin glass tube into the medium (*see* **Note 4**).
5. A maximum yield is achieved with cultures maintained for 8–10 d at 16–18°C (*see* **Note 6**).

3.3. Purification of the Bacteria-Nematode Suspension

The liquid culture will contain in addition to all developmental stages of *C. elegans* also *E. coli* bacteria and fragments of dead worms. Before further treatment, these components must be eliminated.

1. Combine the whole outcome of the 1-L liquid culture in a glass cylinder.
2. Sediment overnight at 4°C.
3. Wash the sediment once with M9 buffer.
4. Spin down at 4500g and 4°C for 3 min.
5. Suspend the pellet in 120 mL M9 buffer.
6. Equally distribute the suspension over eight centrifuge tubes, containing 15 mL suspension each.
7. Add a solution of 15 mL 70% (w/v) saccharose to each tube.
8. Centrifuge for 10 min at 3000g in a swing-out rotor. Bacteria and worm fragments will be contained in the pellet, and living nematodes in the supernatant.
9. Carefully collect the supernatant and wash twice in M9 buffer as described earlier (**step 4**). The pellet gained in this way contains the pure fraction of living nematodes, which is, however, a mixture of adult animals, all developmental stages, and eggs.
10. Freeze the pellet in liquid nitrogen, and store at –80°C for further use (*see* **Note 9**).

3.4. Preparation of Eggs

1. Suspend the nematode pellet from a 1-L liquid culture in 100 mL M9 buffer.
2. Pass the nematode suspension through a nylon net (30-μm mesh size).
3. Adult nematodes will accumulate on the net and are washed with M9 buffer into centrifuge tubes.
4. Spin down at 4500g, 4°C, for 3 min.
5. Slowly stir the pellet in 50 mL of the alkaline hypochloride solution for about 10 min. Under these conditions, the eggs are released from the worms, which is controlled with the use of a microscope (magnification ×400).
6. Centrifuge the suspension at 4500g, 4°C, for 3 min.
7. Wash once the pellet, which contains the released eggs and worm fragments, in M9 buffer as above.
8. Suspend the pellet in 20% (w/v) saccharose and centrifuge at 3000g for 10 min in a swing-out rotor. The supernatant contains the pure egg fraction.
9. Carefully remove the egg fraction.
10. Wash again in M9 buffer.
11. Spin down again as above.
12. Shock-freeze the pellet with liquid N_2, and store at –80°C until use.

3.5. Preparation of Larval Stages L₁–L₄

1. Suspend the nematode pellet from a 1-L liquid culture in 100 mL M9 buffer.
2. Pass the nematode suspension through a nylon net (30-µm mesh size). The fraction that has passed is collected in two centrifuge tubes. It contains the larval stages and a small amount of free eggs. Adult nematodes remain on the net.
3. Spin down at 4500g, 4°C, for 3 min.
4. Transfer the pellet into 15-mL centrifuge tubes, containing 20% (w/v) saccharose.
5. Centrifuge at 3000g for 10 min, in a swing-out rotor. The larvae will be found in the pellet, whereas the few eggs are floating in the supernatant.
6. Discard the supernatant.
7. Wash the pellet in M9 buffer.
8. Freeze the pellet in liquid N$_2$, and store at –80°C until use (*see* **Note 9**).

3.6. Protein Dissolution

The purified nematode, larvae, or egg suspension should be freeze-dried for better storage and handling (mailing, for example).

1. Resuspend approx 5 mg of the lyophilized probe in 1 mL dissolution medium (*see* **Note 10**).
2. Clear the probe by centrifugation.
3. Determine the protein concentration by the Bradford method *(6)*.
4. Adjust the protein concentration with dissolution medium to 0.5 mg/mL.
5. Apply 50 µg protein/100 µL to the Immobiline strip for analytical purposes (*see* Chapters 21–23). The quantity may be varied according to the sensitivity of your staining method. For N-terminal sequence determinations, up to 1 mg protein is loaded on a strip by repeatedly applying 100 µL of the probe until the desired quantity was reached.

4. Notes

1. *C. elegans* can be maintained for prolonged periods on 9.4-cm agar plates, carrying a lawn of the *E. coli* strain OP 50, which is auxotroph for uracil (for details, *see* **refs.** *1* and *3*).
2. Growth on *E. coli* is to be preferred also for liquid cultures: it would be much less reliable and much more expensive to grow worms axenically. For liquid cultures, any fast-growing, wild-type of *E. coli* is equally suited.
3. Be sure to maintain sterile conditions throughout the whole duration of the culture. Avoid taking probes. If necessary, observe strictly aseptic precautions. Shake first, because the nematodes are sedimenting rapidly.
4. The densely growing liquid culture of *C. elegans* has a high oxygen requirement. Effective ventilation under sterile conditions is therefore crucial.
5. The growing worm population excrets ammonia. If the pH rises considerably above 8.0, the population may die suddenly. The phosphate buffer counteracts this effect.
6. Harvest before a large portion of larvae enters into dauer stage.

7. The number of free eggs in the culture medium is very low. For this reason, the release of eggs from the nematodes is stimulated chemically, which leads to a satisfactory yield.

8. What are called "eggs" in the literature are actually embryonic larval stages. The nematode starts its development on self-fertilization already in the ovaries.

9. Deep-freezing is suggested only for probes from which proteins or DNA will be extracted. L_1 and L_2 larvae can be stored indefinitely at –80°C in freezing solution. For details *see* **refs. 2**.

10. For a very effective rupture of the nematode cuticula and protein solubilization, the application of the "dissolution medium" is completely sufficient. No prior treatment is needed.

Acknowledgments

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7

Eukaryotic Cell Labeling and Preparation for 2-D

Nick Bizios

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2-DE) provides the ability to resolve and quantify thousands of proteins from an unfractionated eukaryotic cell lysate. The use of 2-DE in studying eukaryotic cells has run the gambit from ozone-stressed Norway spruce needles *(1)*, mapping proteins in the differentiation of mouse endoderm, mesoderm, and ectoderm *(2)* to the study of apoptosis *(3)*.

Many parameters and conditions influence the resolution of proteins on 2-DE, such as the pH range of carrier ampholytes used, the quality of reagents and equipment used, temperature, voltage, and the skill of the researcher or technician. However, the radiolabeling of eukaryotic cells and their subsequent preparation for 2-D PAGE may be considered paramount in obtaining consistent results. Without proper radiolabeling and preparation, how would one properly *(1)* find subsequent spots of interest and *(2)* perform quantitative analysis?

This chapter will describe a general method for labeling methionine-containing proteins, phosphorylation labeling, and subsequent lysate preparation for 2-DE that has been modified from Garrels *(4)* and Garrels and Franza *(5)*. The Jurkat T-lymphoblast cell line is used as an example.

A large number of protocols have been published for the solubilization and sample preparation of eukaryotic cell lines and tissues for 2-DE. One of the best places for additional protocols is found at the Geneva University Hospital's Electrophoresis Laboratory, which can be accessed via the WWW at http://expasy. hcuge.ch/ch2d/technical-info.html.

2. Materials

2.1. Equipment

1. 0.2-μm Filters.
2. Heat block or 100°C water bath.

2.2. Reagents

1. Complete culture medium: 90% RPMI-1640, 10% fetal bovine serum (FBS), streptomycin-penicillin: Mix 990 mL of RPMI-1640 with 100 mL of FBS, and add 10 mL of streptomycin-penicillin (100X). Cold-filter-sterilize using a 0.2-μm filter. Store at 4°C. Maintain the Jurkat T-lymphoblasts at a concentration of 10^5–10^6 cells/mL (*see* **Note 1**).
2. Methionine-free medium: 90% methionine-free RPMI-1640, 10% dialyzed FBS (dFBS): Mix 990 mL of methionine-free RPMI-1640 with 100 mL of dFBS. Cold-filter-sterilize using a 0.2-μm filter. Store at 4°C.
3. Sodium phosphate-free medium: 90% sodium phosphate-free RPMI-1640, 10% FBS: Mix 90% sodium-phosphate free RPMI with 10% FBS. Cold-filter-steril-ize. Store at 4°C.
4. ^{35}S-label (EXPRE$^{35}S^{35}S$[^{35}S] methionine/cysteine mix (New England Nuclear [NEN]).
5. ^{32}P-orthophosphate (NEN).
6. Phosphate-buffered saline (PBS): Mix 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of dH_2O, and adjust pH to 7.4 with HCl. Add dH_2O to a final volume of 1000 mL and autoclave. Store at room temperature.
7. Dilute SDS (dSDS): 0.3% SDS, 1% β-mercaptoethanol (β-ME), 0.05 *M* Tris-HCl, pH 8.0. In a cold room, mix 3.0 g of SDS, 4.44 g of Tris-HCl, 2.65 g of Tris base, 10 mL of β-ME in distilled water, and adjust the final volume to 1 L with dH_2O. Aliquot 500 μL into microcentrifuge tubes, and store at –70°C.
8. DNase/RNase solution: 1 mg/mL DNase I, 0.5 mg/mL RNase A, 0.5 *M* Tris, 0.05 *M* $MgCl_2$, pH 7.0. Thaw RNase, Tris, and $MgCl_2$ stocks, and thoroughly mix 2.5 mg of RNase A (Worthington Enzymes), 1585 μL of 1.5 *M* Tris-HCl, 80 μL of 1.5 *M* Tris base, 250 μL of 1.0 *M* $MgCl_2$, and 2960 μL of dH_2O. Mix the liquids, and add 5 mg of DNase I (Worthington Enzymes). Do not filter. Keep cool while dispensing into microcentrifuge tubes. Make 50-μL aliquots, and store at –70°C.
9. 1.5 *M* Tris-HCl solution: Weigh out 11.8 g of desiccated Tris-HCl, and add 41.4 g of dH_2O. Mix well and filter through 0.2-μm filter. Aliquot into microcentrifuge tubes, and store at –70°C.
10. 1.5 *M* Tris base solution: Weigh out 9.09 g of Tris base, and add 41.4 g of dH_2O. Mix and filter through 0.2-μm filter. Aliquot into microcentrifuge tubes, and store at –70°C.
11. 1.0 *M* $MgCl_2$ solution: Weigh out 30.3 g of $MgCl_2$, and add 85.9 g of dH_2O. Mix and filter through 0.2-μm filter. Aliquot into microcentrifuge tubes, and store at –70°C.
12. Sample buffer solution (SB): 9.95 *M* urea, 4.0% Nonident P-40 (NP40) (Sigma), 2% pH 6.0–8.0 ampholytes, 100 m*M* dithiothreitol (DTT). Mix 59.7 g of urea,

44.9 g of dH$_2$O, 4.0 g of NP40, 5.5 g of pH 6.0–8.0 ampholytes (Pharmacia), 1.54 g of DTT (Calbiochem) in this order in a 30- to 37°C water bath just long enough to dissolve the urea. Filter through a 0.2-µm filter and aliquot 1 mL into microcentrifuge tubes. Snap-freeze in liquid nitrogen, and store at –70°C.

13. Sample buffer with SDS solution (SBS): 9.95 *M* urea, 4.0% NP40, 0.3% SDS, 2% pH 6.0–8.0 ampholytes, 100 m*M* DTT. Mix 59.7 g of urea, 44.9 g of dH$_2$O, 4.0 g of NP40, 0.3 g of SDS, 5.5 g of pH 6.0–8.0 ampholytes, 1.54 g of DTT in this order in a 30- to 37°C water bath just long enough to dissolve the urea. Filter through a 0.2-µm filter, and aliquot 1 mL into microcentrifuge tubes. Snap-freeze in liquid nitrogen, and store at –70°C.

3. Methods
3.1. ^{35}S-Labeling (see *Note 2*)

1. Jurkat T-lymphocytes are labeled for 3–24 h in methionine-free media containing 50–250 µCi/mL of ^{35}S.
2. Follow cell lysate protocol (**Subheading 3.3.**).

3.2. ^{32}P-Labeling (see *Note 2*)

1. Add 100 µCi/mL of ^{32}P for up to 3 h to cells that are in phosphate-free medium (*see* **Note 3**).
2. Follow cell lysate protocol (**Subheading 3.3.**).

3.3. Whole-Cell Lysate Preparation

1. Wash cells with PBS three times in a microcentrifuge tube.
2. Add an equal volume of hot (100°C) dSDS solution to the pellet.
3. Boil tube (100°C) for 1–3 min.
4. Cool in an ice bath (*see* **Note 4**).
5. Add one-tenth ($^1/_{10}$) volume of DNase/RNase solution.
6. Gently vortex for several minutes to avoid foaming. The sample should lose its viscosity, and the solution should look clear. If not, then add more dSDS and DNase/RNase solution (*see* **Note 5**).
7. Snap-freeze in liquid nitrogen, and store at –70°C. Samples may be kept for up to 6 mo at –70°C.

3.4. Preparing the Sample for 2D-PAGE
3.4.1. Vacuum Drying

1. Lyophilize sample (frozen at –70°C) in a Speed Vac using no or low heat until dry.
2. Add SB solution to the sample equal to that of the original dSDS sample volume, and mix thoroughly.
3. Heat sample to 37°C for a short period if necessary (*see* **Note 6**).
4. Store at –70°C. Samples can be kept for up to 6 mo at –70°C.
5. Radioisotope incorporation in the sample may now be determined by TCA precipitation (*see* Chapter 13).

6. Recommended first-dimension load is 500,000 dpm for ^{35}S-labeled proteins and 200,000 dpm for ^{32}P-labeled proteins.
7. If necessary, the sample is diluted and mixed thoroughly with SBS solution before loading onto the first-dimension gel (*see* **Notes 6**).

4. Notes

1. Maintain Jurkat T-lymphocytes in complete culture medium supplemented with streptomycin/penicillin, in a humidified incubator with 95% air and 5% CO_2 at 37°C and at a concentration of 10^5–10^6 cells/mL.
2. It is extremely important that all radioactive work be performed with the utmost care, and according to your institutional and local guidelines.
3. It may be necessary to preincubate the cells. Preincubating the cells at a density of 1–10×10^6 cells/mL for 30 min in sodium-free, phosphate-free medium works well.
4. Sample preparation should be done quickly on ice to avoid degradation by proteases.
5. Keep salt concentrations as low as possible. High concentrations (>150 m*M*) of NaCl, KCl, and other salts cause streaking problems, as do lower concentrations of phosphate and charged buffers. Dialyzing samples to remove salts and other low-mol-wt substances is recommended.
6. After dissolving the sample in SB solution or diluting in SBS solution, it is imperative that the sample is not subjected to temperatures above 37°C. At extreme temperature (>40°C), the urea in SB and SBS will cause carbamylation. Charged isoforms will be generated by isocyanates formed by the decomposition of urea.

Acknowledgment

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8

Differential Detergent Fractionation of Eukaryotic Cells

Analysis by Two-Dimensional Gel Electrophoresis

Melinda L. Ramsby and Gregory S. Makowski

1. Introduction

Differential detergent fractionation (DDF) represents an alternative method for cell fractionation that employs sequential extraction of cells or tissues with detergent-containing buffers to partition cellular proteins into structurally and functionally intact and distinct compartments *(1–5)*. Relative to cell fractionation by differential pelleting, DDF has the advantage of preserving the integrity of microfilament and intermediate filament cytoskeletal networks, and is especially applicable to use with limited quantities of biomaterial *(4–6)*. In addition, DDF is simple, highly reproducible, labor-sparing, and ultracentrifuge-independent. DDF is appropriate for a variety of investigations *(7–15)*, including those objecting to:

1. Enhance the delectability of low-abundance species or semipurify components of known subcellular localization.
2. Define the subcellular localization of enzymes, regulatory, or structural proteins as well as nonprotein metabolites.
3. Monitor physiologic fluxes and compartmental redistribution of biomolecules under basal and stimulated conditions.
4. Identify cytoskeletal-associated and interacting proteins.
5. Investigate the role of cytoskeletal networks in the subcellular localization of endogenous and exogenous factors, including mRNA, viral components, and heat shock proteins, interactions relevant to understanding mechanisms of infection, protein turnover, and the stress response.

The DDF protocol detailed here reproducibly partitions cellular proteins into distinct cytoskeletal and noncytoskeletal compartments that can be directly analyzed by two-dimensional (2-D) gel electrophoresis. This straightforward
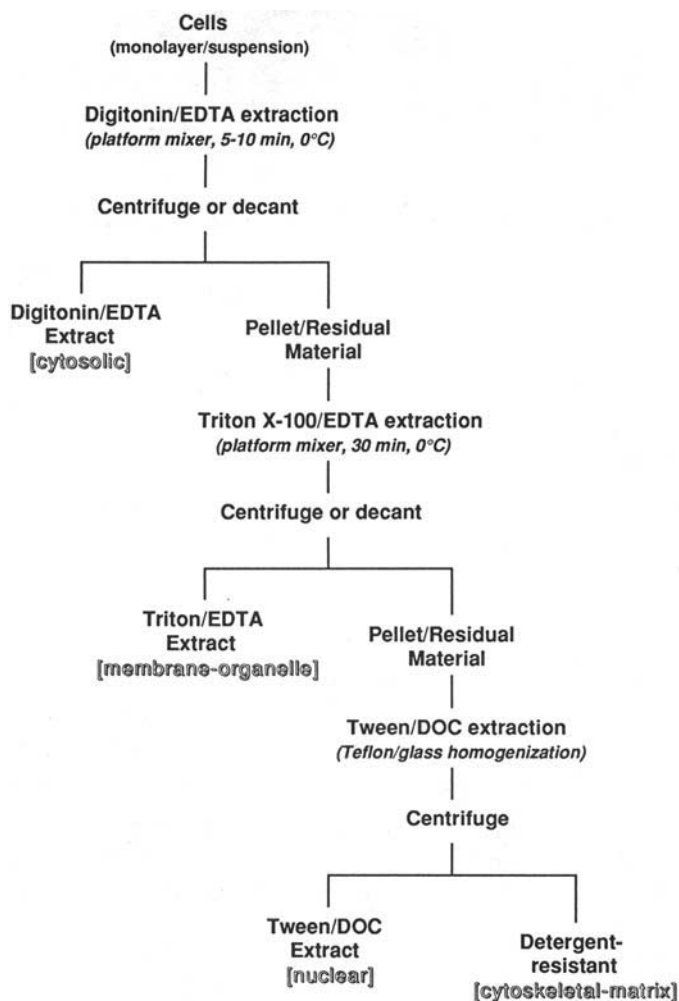
Fig. 1. Simplified schematic for DDF of cells cultured in suspension or in monolayers (*see* **Subheading 3.** for details).

methodology is applicable to a variety of studies and cell types relevant to basic and clinical research. Overall, the protocol entails the sequential extraction of cells (suspension cultures or monolayers) with three detergent-containing buffers:

1. Digitonin/EDTA.
2. Triton X-100/EDTA.
3. Tween-40/deoxycholate (*see* **Fig. 1**).

All solutions are pH-adjusted at 4°C and contain the organic buffer PIPES, as well as the neutral serine protease inhibitor PMSF.

DDF reproducibly yields four electrophoretically distinct fractions, which contain:

1. Cytosolic proteins and soluble cytoskeletal elements.
2. Membrane and organellar proteins.
3. Nuclear membrane and soluble nuclear proteins.
4. Detergent-resistant cytoskeletal filaments with nuclear matrix proteins (*see* **Fig. 2** and **Table 1**).

Biochemical, immunochemical, and electrophoretic characterization of these fractions were recently described *(16)*. We have used this procedure to fractionate successfully a variety of cell types incubated in suspension culture (hepatocytes, neutrophils) or grown as monolayers (corneal and vascular endothelial cells, normal and malignant osteoblasts, fibroblasts). In addition, we have been able to analyze zymographically the intracellular and extracellular localization of fibrinolytic enzymes *(17)*, as well as, purify intact RNA from each fraction for studies of mRNA distribution and turnover (manuscript in preparation). Thus, the potential applications of this method are numerous.

2. Materials

2.1. Equipment

1. Ultrapure water (double-distilled, deionized, >18Ω) is used for all reagent preparations.
2. Reagent grades should be the highest quality as appropriate for intended use (cell culture, molecular biology, ultrapure electrophoretic).

2.2. Reagents

2.2.1. Stock Solutions

1. 100 mM Ethylenediaminetetraacetic acid (EDTA) stock solution: Dissolve 3.36 g EDTA in 100 mL water (final volume). Store at room temperature.
2. 100 mM Phenylmethylsulfonyl fluoride (PMSF) stock solution: Dissolve 174 mg PMSF in 10 mL isopropanol. Sonicate if necessary. Store at room temperature in the dark.
3. Piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) stock buffers (4 and 10X):

Reagent	FW, g/mol	4X Stock buffer per 250 mL, g	mM (1X)	10X Stock buffer per 100 mL, g	mM (1X)
Sucrose	342	103	300	—	—
NaCl	58.4	5.8	100	0.58	10
PIPES	302	3	10	3	10
MgCl$_2$ · 6H$_2$O	203	0.64	3	0.20	1

Filter through a 0.45-μm sterile filter. Store at 4°C in dark.
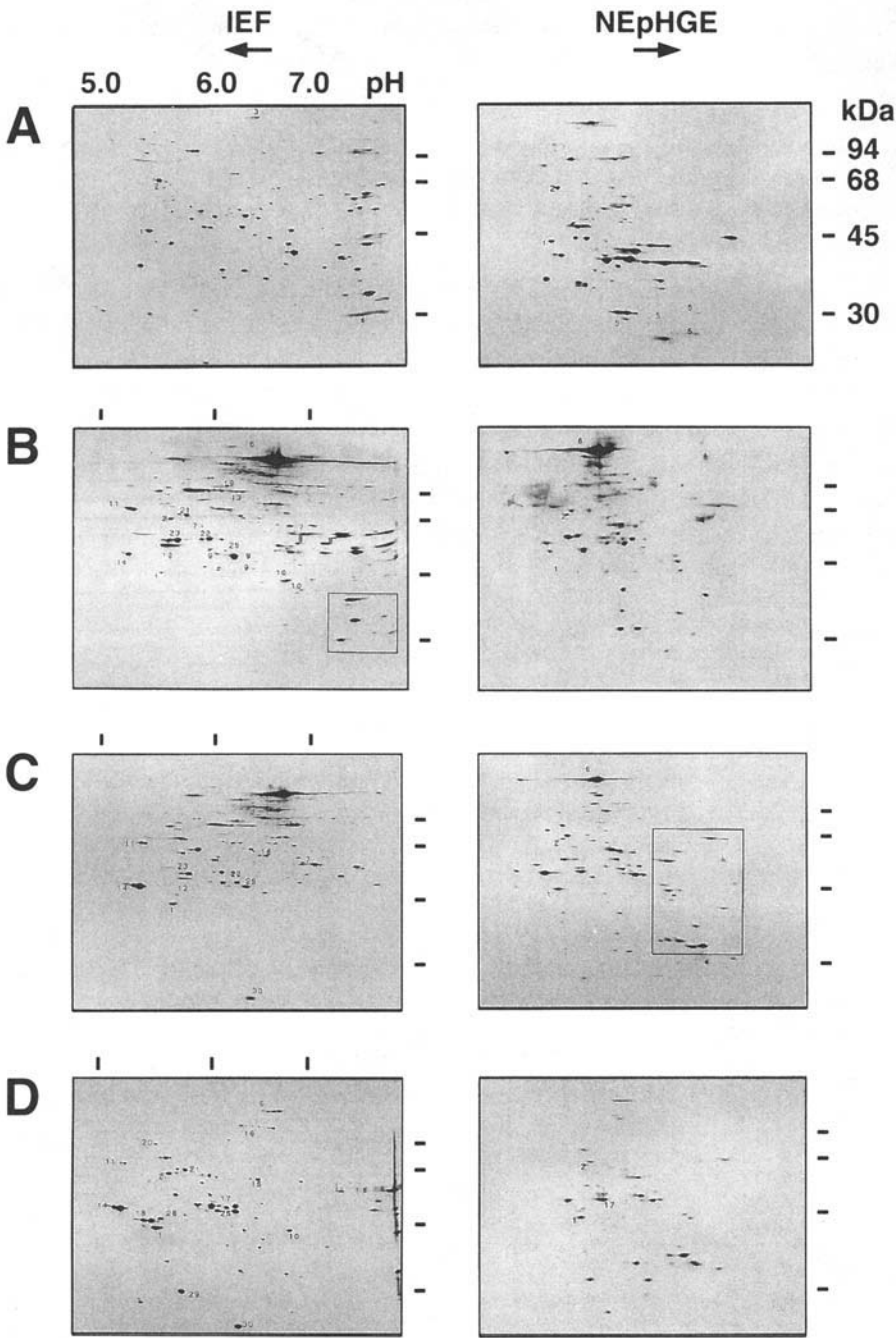
Fig. 2. Silver-stained 2-D IEF and NEpHGE gels of DDF obtained from suspension-cultured rat hepatocytes. DDF extracts: **(A)** digitonin/EDTA (cytosolic); **(B)**

2.2.2. Detergent Extraction Buffers (see **Note 2**)

1. Digitonin extraction buffer: 10 mM PIPES, pH 6.8, at 4°C, 0.015% (w/v) digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl$_2$, 5 mM EDTA, 1 mM PMSF. Dissolve by heating 18.75 mg digitonin in 10 mL 4X stock buffer in a small flask with a stir bar, and then add 1 mL PMSF. Combine with remaining reagents: 15 mL 4X stock buffer and 5 mL EDTA (*see* **Note 3**). Add water to 100 mL (final volume).

2. Triton X-100 extraction buffer: 10 mM PIPES, pH 7.4, at 4°C, 0.5% (v/v) Triton X-100, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl$_2$, 5 mM EDTA, 1 mM PMSF. Combine 25 mL 4X stock buffer, 1 mL PMSF, 3 mL EDTA, and 5 mL freshly prepared 10% Triton X-100 (*see* **Note 4**). Add water to 100 mL (final volume).

3. Tween-40/deoxycholate extraction buffer: 10 mM PIPES, pH 7.4, at 4°C, 1% (v/v) Tween-40, 0.5% (v/v) deoxycholate, 1 mM MgCl$_2$, 1 mM PMSF. Separately dissolve 0.5 g DOC in 2.5 mL 10X stock buffer and 1 mL Tween-40 in 2.5 mL 10X stock buffer (warm to dissolve if necessary). Combine and add 5 mL 10X stock buffer and 1 mL PMSF (*see* **Note 5**). Add water to 100 mL (final volume).

4. Cytoskeleton solubilization buffers: (a) Nonreducing buffer—5% (w/v) SDS, 10 mM sodium phosphate, pH 7.4. Solubilize 0.5 g SDS in 5 mL 20 mM sodium phosphate buffer, pH 7.4. Add water to 10 mL (final volume). (b) Denaturing buffer—5% (w/v) SDS, 10 mM sodium phosphate, pH 7.4, 10% (v/v) β-mercaptoethanol. Add 1 mL β-mercaptoethanol to recipe. Adjust water appropriately.

2.2.3. Sample Lysis Buffers

1. O'Farrell lysis buffer (1X): 9.5 M urea, 2% (v/v) NP40, 1.6% ampholines pH 5.0–7.0, 0.4% ampholines pH 3.0–10.0, 5% (v/v) β-mercaptoethanol. Dissolve 5.7 g ultrapure electrophoretic-grade urea, 0.1 mL NP40, 0.2 mL ampholines (0.16 mL pH 5.0–7.0 and 0.04 mL pH 3.0–10.0), 0.5 mL β-mercaptoethanol in ultrapure water. Bring to 10 mL final volume with water. Solution may be warmed to facilitate solubilization. Aliquot (1 mL) and store at –70°C.

2. O'Farrell lysis buffer (10X): 20% (v/v) NP40, 16% ampholines pH 5.0–7.0 4% ampholines pH 3.0–10.0, 50% (v/v) β-mercaptoethanol. Combine 0.2 mL NP40, 0.2 mL ampholines (0.16 mL pH 5.0–7.0 and 0.04 mL pH 3.0–10.0), 0.5 mL β-mercaptoethanol in water. Bring to 1 mL final volume with water. Solution may be warmed to facilitate solubilization. Aliquot (100 μL) and store at –70°C.

Triton X-100/EDTA (membrane-organelle); **(C)** Tween-40/DOC (nuclear); and **(D)** detergent-resistant (cytoskeletal-matrix) fraction. Isoelectric point (pI) values shown at top and mol-wt (kDa) values shown on left (reproduced with kind permission from VCH Publishers; for details, *see* **ref. *16***).

Table 1
Subcellular Protein Distribution in Differential Detergent Fractions[a]

	Detergent fraction			
Constituent	Digitonin/EDTA, cytosolic	Triton/EDTA, membrane-organelle	Tween/DOC, nuclear	Detergent resistant, cytoskeletal
Protein (% of total)	35	50	5	10
Marker enzymes[b] (% of total)	LDH (90) GST (84)	β-Gal (92) GDH (98) cyto P450 (92) 5'-nucleotidase (73)	5'-nucleotidase (18)	
Noncytoskeletal proteins	Carbonic anhydrase Acetyl CoA carboxyl hsp-70 hsp-47 Fatty acid bind prot. Calpain I and II Calpastatin	Pyruvate dehydrog. Carbamoyl. phos. synth. Cyto b_5 reductase GRP-78 Carnit palmit transf. Prot. Disulf. isom. α-Connexin	p38 Nucl. prot.	Ribophorin Docking prot
Cytoskeletal proteins	Desmoplakin II Myosin Vinculin α-Tubulin β-Tubulin Actin	Actin	Actin	Desmoplakin I Desmoplakin II Vinculin Nuclear lamins Cytokeratin A/D Actin

[a]Distribution profiles were obtained by marker enzyme analysis or immunoblotting. These distributions reflect the fractionation profile for hepatocytes (16), a highly metabolic cell type. Fractionation profiles may vary with less metabolically active or specialized cell types.

[b]LDH, lactate dehydrogenase; GST, glutathione-S-transferase; hsp-70, heat-shock protein-70; β-Gal, β-galactosidase; GDH, glutamate dehydrogenase; cyto P450 red, NADPH cytochrome P450 reductase; GRP-78, glucose-regulated protein-78 (for details, *see* **ref. 16**).

3. Methods

3.1. Cell Preparation

DDF can be employed to fractionate cells cultured in suspension or in monolayers. Following DDF, all extracts are stored frozen at –70°C. Save an aliquot of DDF buffers at –70°C to use for sample normalization and as a control material in enzymatic and protein analyses (*see* **Subheadings 3.3.** and **3.4.2.**). To eliminate culture media effects, cells should be washed twice in ice-cold saline, PBS, or other nondetergent buffer prior to DDF.

3.1.1. Suspension-Cultured Cells

The volume of DDF solutions for suspension-cultured cells is based on wet weight or cell number. To determine wet weight, transfer an aliquot of suspension culture to a preweighed plastic tube and centrifuge briefly. Decant the culture media, and determine wet weight of the cell pellet. Digitonin/EDTA extraction buffer (5 vols/g wet wt) can be added directly to cell pellets. For volumes of other DDF buffers, *see below*.

3.1.2. Monolayer-Cultured Cells

The volume of DDF buffers for monolayer cell cultures is determined per surface area or cell number. For a typical T_{25} culture flask (~5 × 10^6 cells), 1 mL of digitonin/EDTA buffer is used initially. Following removal of culture media, DDF can be performed in the culture flask. For volumes of other DDF buffers, *see below*.

3.2. Detergent Fractionation (see Note 6)

3.2.1. Digitonin Extraction (Cytosolic Fraction)

1. Add ice-cold digitonin extraction buffer to washed cell pellets (5 vols/g wet wt, gently resuspend by swirling) or monolayers (1 mL/T_{25} flask) (*see* **Notes 7** and **8**).
2. Incubate cells on ice with gentle agitation (platform mixer) until 95–100% of cells are permeabilized (5–10 min) as assessed by trypan blue exclusion.
3. For suspension-cultured cells, centrifuge the extraction mixture (480*g*), and remove supernatant. For cell monolayers, tilt the culture flask, and remove extract (cytosolic proteins) with a pipet.
4. Record extract volume, aliquot, and store at –70°C.

3.2.2. Triton X-100 Extraction (Membrane/Organelle Fraction)

1. Carefully resuspend digitonin-insoluble pellets in ice-cold Triton X-100 extraction buffer in a volume equivalent to that used for digitonin extraction (5 vol relative to starting wet weight) to obtain a homogeneous suspension (*see* **Note 9**).
2. For monolayer cultures, add 1 mL Triton X-100 extraction buffer/T_{25} flask equivalent (~5 × 10^6 cells).

3. Incubate on ice with gentle agitation (platform mixer) for 30 min.
4. Remove Triton extract (membrane and organellar proteins) by centrifuging suspensions (10 min, 5000g), or tilting and decanting monolayers.
5. Measure volume of extract, aliquot, and store at –70°C.

3.2.3. Tween/DOC Extraction (Nuclear Fraction)

1. Resuspend the Triton-insoluble pellets from suspension cultures in Tween/DOC extraction buffer at one-half the volume used for Triton extraction; resuspend using a Teflon smooth-walled glass homogenizer (five strokes, medium speed) (*see* **Note 10**).
2. Remove Tween/DOC extract (nuclear proteins) by pelleting detergent-resistant residue (6780g).
3. Extract cell monolayers with 0.5–1 mL Tween/DOC buffer/T$_{25}$ flask equivalent.
4. Record volume, aliquot, and store at –70°C.

3.2.4. Detergent-Resistant Residue (Cytoskeletal/Nuclear Matrix Fraction)

1. The detergent-resistant pellet is washed in ice-cold PBS (pH 7.4, 1.2 mM PMSF) by resuspension (Teflon/glass homogenizer) and centrifugation (12,000g) to shear DNA mechanically (*see* **Note 11**).
2. Pellets from suspension cultures are washed once with –20°C 90% acetone, lyophilized, and weights determined in tared Eppendorf centrifuge tubes. Samples are stored at –70°C.
3. Monolayers are rinsed *in situ* with PBS, and the detergent-resistant residue is suspended directly into nondenaturing cytoskeleton (CSK) solubilization buffer without β-mercaptoethanol by tituration. Store at –70°C.

3.3. Protein Determination

1. Thaw aliquots of detergent buffers and detergent extracts on ice.
2. Dilute an aliquot of digitonin and Triton X-100 extracts with 4 vol of ultrapure water (extracts obtained from monolayer culture may require less dilution). The Tween/DOC extract is used without dilution.
3. Solubilize lyophilized CSK pellets in CSK solubilization buffer minus β-mercaptoethanol (10 mg dry wt/mL). CSK preparations from monolayers are diluted as necessary.
4. Assay 20–50 μL of diluted or undiluted sample from each fraction, in duplicate, using the Folin-phenol method of Peterson *(18)* (*see* **Note 12**). Use oven-dried bovine serum albumin prepared in each detergent buffer to generate standard curves.

3.4. 2-D Gel Electrophoretic Analysis

In our experience, DDF samples obtained from a variety of cell types can be utilized for 2-D PAGE under both isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEpHGE). Samples to be compared by 2-D gel analysis are first normalized to contain equal protein in equal volumes (*see* example in **Subheading 3.4.2.**).

3.4.1. Sample Preparation

1. Fresh or defrosted DDF samples are kept on ice. Samples are obtained from digitonin, Triton X-100 and Tween-40/DOC extracts are brought to 9.5 *M* urea by addition of solid urea. For 100 µL sample, add 85 mg urea and 15 µL 10X O'Farrell lysis buffer, and warm to room temperature (*see* **Note 13**).
2. For the dried CSK extract, solubilize directly in 1X O'Farrell lysis buffer *(19,20)*.
3. For CSK extracts in nonreducing SDS buffer, bring to 9.5 *M* urea by addition of solid urea and add 10X lysis buffer.

3.4.2. Sample Normalization

Samples for comparison are normalized with respect to protein concentration prior to addition of lysis buffers or urea by volume normalization using the appropriate detergent extraction buffer. For example, to normalize three digitonin/EDTA samples containing protein at 8, 9, and 10 µg/µL make the following additions:

Sample	Sample, µL	Digitonin/EDTA buffer, µL	Urea, mg	10X Lysis, buffer, µL	Load, µL
1	100	0	85	15	20
2	90	10	85	15	20
3	80	20	85	15	20

3.4.3. 2-D Electrophoresis

DDF samples are subjected to 2-D gel electrophoresis by established methods *(19,20)* (*see* **Note 14**). IEF gels contain a total of 3.5% ampholines (2% pH 5.0–8.0, 1% pH 3.0–10.0, 0.5% pH 2.0–5.0) and samples are electrophoresed for a total of 9800 V/h with hyperfocusing at 800 V for the final hour *(16,21)*. NEpHGE gels contain 2%, pH 3.0–10.0, ampholines, and samples are electrophoresed for 2400 V/h *(16)*.

4. Notes

1. For 4X stock solutions, solubilize sucrose and NaCl together in water. Solubilize PIPES separately in a small volume of 1 *M* NaOH before mixing with remaining ingredients. MgCl$_2$ can be added directly to sucrose solution or final buffer as a solid or from a concentrated stock solution prepared in ultrapure water, as convenient. Stock buffers should be sterile filtered (0.45 µm) and stored at 4°C in the dark. Stock solutions are stable for up to 2 mo. Maintain aseptic technique when diluting stock for preparation of 1X working solutions. Alternately, stock buffer solutions may be stored in volumes appropriate for single-use aliquots.
2. Stability of detergent extraction buffers varies. Digitonin solutions are reportedly stable in buffer for approx 3 h at 0°C *(22)*. Triton X-100 and Tween-40 are nonionic detergents and decompose in aqueous buffer to form peroxide radicals,

which oxidize sulfhydryl groups *(1,23,24)*. In our experience, 1X working digitonin and Triton X-100 buffers if prepared with EDTA remain stable longer, and can be aliquoted and fresh-frozen at –70°C. Such solutions should be thawed on ice and not refrozen.

3. The 1X working digitonin extraction buffer should be prepared by adding solid digitonin to a small amount of buffer and carefully boiling to dissolve. PMSF is solubilized in the warm digitonin solution by slow addition with constant stirring (magnetic stir bar). The digitonin/PMSF solution is then added to the remaining buffer ingredients. The solution is cooled to 4°C, and the pH adjusted to 6.8 with dilute HCl. The digitonin extraction solution is brought to volume with water and kept on ice until use or aliquoted, and stored frozen at –70°C as noted above.

4. The 1X working Triton X-100 extraction buffer should be prepared using freshly made 10% Triton X-100 in ultrapure water. The solution is cooled to 4°C, pH adjusted to 7.4, and brought to final volume. The solution is kept on ice or stored frozen as noted above.

5. The 1X working Tween/DOC buffer is prepared by dissolving Tween-40 and DOC separately in a small volume of buffer with heating to dissolve. The solutions are mixed, cooled to 4°C, pH adjusted to 7.4, and brought to volume. The solution is used fresh. Unused buffer is discarded.

6. The extraction protocol described here represents a modification of a method described by Fey et al. for fractionation of MDCK cells *(4)*. Modifications include the addition of a digitonin extraction step, the inclusion of EDTA in digitonin and Triton buffers, and the exclusion of a nuclease digestion step (DNA is denatured by shear force in the presence of SDS).

7. The digitonin extraction protocol was formulated in accord with considerations described in the literature *(22,25–27)*. Briefly, digitonin is a steroidal compound believed to complex with plasma membrane cholesterol, resulting in membrane permeabilization and the rapid release of soluble cytosolic components leaving behind intact cell ghosts and heavy organelles. At concentrations of 0.015%, digitonin preserves the ultrastructure of ER and mitochrondrial membranes *(26)*, which at higher concentrations (~0.1%) are damaged secondary to solubilization of membrane phospholipid *(26,27)*.

   In our experience, the inclusion of EDTA significantly enhances the effectiveness of low concentrations of digitonin, as evidenced by an increased rate of membrane permeabilization (10 min +EDTA vs 40 min –EDTA). In addition, EDTA is beneficial for inhibiting calcium-dependent neutral proteases, typically enriched in cytosolic extracts *(16)* and, thus, avoids artifactual proteolysis. Consistent with the reports of others, digitonin releases proteins larger than 200 kDa *(27)*, as evidenced by the presence of myosin (>220 kDa), desmoplakin II (>250 kDa), and the calpain-inhibitor calpastatin (~300 kDa) in cytosolic extracts *(16)*.

   In brief, digitonin/EDTA extraction as described here, yields a cytosolic extract representing ~35% total cellular protein, which is enriched in cytosolic markers (90% LDH activity, 100% carbonic anhydrase immunoreactivity) and essentially devoid of mitochrondrial, lysosomal, and ER markers *(16)*; it there-

fore represents a significant improvement over previous low concentration digitonin extraction methods, which released only 60% of LDH concomitant with ~30% contamination from organelle markers *(10,28,29)*.

8. The selective fractionation of cytoskeletal tubulins in the digitonin extract likely reflects the effect of both cold and EDTA on inducing microtubule depolymerization, and has been capitalized on as a first-step method for preparing polymerization-competent microtubules (unpublished investigations).

9. Triton is a nonionic detergent that solubilizes membrane lipids and releases organelle contents. It has been used in hyper- or hypotonic buffers to prepare cytoskeletal preparations enriched in intermediate filaments *(30)*. Lower concentrations of Triton concomitant with isoosmolar, isotonic buffer composition preserves nuclear and microfilament integrity *(4)*. Thus, as used here, Triton extracts are enriched in markers for membrane and organelle proteins *(16)* and constitute the bulk of cellular proteins (~50% total protein). Triton extraction is possible using either X-100 or X-114 series. Fractionation with X-114 allows subfractionation of peripheral vs integral membrane proteins *(31,32)* and lends further flexibility to fractionation goals.

10. DOC is a weakly ionic detergent that destroys nuclear integrity *(33)*, and solubilizes actin and other cytoskeletal elements *(4,5)*. Tween/DOC buffer extracts ~5% of total cell protein and contains exclusively, immunoreactivity for the nuclear protein p38 *(16)*, thereby verifying that nuclear integrity persists through Triton extraction. Consideration of marker enzyme profiles (*see* **Table 1**), Western blot analysis, and 2-D electrophoretograms *(16)* suggests that although Tween/DOC buffer extracts proteins common to both the membrane/organelle and detergent-resistant cytoskeletal fractions, specific distinctions are apparent, suggesting this fraction represents a metabolically distinct, possibly more labile, protein compartment. However, for studies in which nuclear parameters are not of interest, the DDF protocol may be simplified by omitting the Tween/DOC step; this may be especially warranted when fractionating limited amounts of sample.

11. The detergent-resistant fraction accounts for ~7–10% of cellular protein and is enriched in intermediate filaments, actin, and various cytoskeletal associating/interacting proteins *(16)*. It also contains the nuclear matrix proteins and DNA. DNA causes a viscous, difficult-to-manage extract, but can be readily denatured by mechanical shear *(34)* using either Teflon/glass homogenization (for suspension pellets) or tituration with a pipet (for monolayer residues). This fraction is intact as evidenced by the absence of staircase patterns (indicative of proteolytic degradation) on 2-D PAGE. Staircase patterns are obtained if PMSF or EDTA are absent from extraction buffers. Solubilization of detergent-resistant samples in SDS-containing phosphate buffer in the absence of mercaptoethanol allows direct assay of protein content by the method of Peterson *(18)*.

12. The Folin-phenol method of Peterson has been extensively detailed elsewhere *(18)*. Briefly, this method is considered the method of choice for international laboratory standardization of protein values and is not susceptible to interference by detergents, thus enabling direct analysis of samples. In contrast, assay by the

standard Lowry method results in detergent-induced flocculation (personal observation). Direct analysis avoids the cumbersome practice of protein precipitation prior to analysis as was used in earlier detergent fractionation protocols *(4)*. We have verified the lack of detergent interference by comparing blanks and standards prepared in detergent buffers at various dilution in triplicate assays.

13. Soluble DDF extracts (digitonin, Triton, and Tween/DOC) may contain low concentrations of protein. To minimize the dilutional effects of the lysis buffer, solid urea and 10X O'Farrell lysis buffer are added to these samples. The sample may be warmed slightly to facilitate urea solubilization. **Caution:** excessive warming (increased temperature and/or prolonged heating) may result in carbamylation artifacts.

14. Typically, 25–100 µg protein in 15–60 µL provide adequate sample for visualization by Coomassie blue or autoradiography. Lesser concentrations may be analyzed by silver staining. In our experience, the volume of detergent buffer contained in samples of these volumes did not adversely affect the linear range of the pH gradient in IEF gels. It should be noted, however, that a slight shift to more acidic values may occur with DDF samples containing Tween/DOC.

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9

Fractionated Extraction of Total Tissue Proteins from Mouse and Human for 2-D Electrophoresis

Joachim Klose

1. Introduction

The protocol for extracting proteins from mouse and human tissues (organs) described in this chapter follows a strategy that is based on the intention to include all the various protein species of a particular tissue in a set of samples that are suitable for two-dimensional electrophoresis (2-DE), particularly for the large gel 2-DE technique described in Chapter 18. The aim then is the resolution and visualization of all these protein species in 2-DE gels. This aim explains some features of our tissue extraction procedure for gaining the proteins. Fractionation of total tissue proteins was preferred to a one-step extraction of all proteins. Using the fractionation procedure, the many different protein species of a tissue can be distributed over several 2-DE gels, and this increases resolution. However, a postulate is that fractionation of tissue proteins results in fraction-specific proteins. To achieve this, cell fractionation is usually performed with the aim of isolating special cell organelles (nuclei, mitochondria) or cell structures (membranes). The proteins are then extracted from these natural fractions. This procedure, however, includes washing steps to purify the cell fractions, and the elimination of cell components that are not of interest and cell residues, which are rejected. Using this procedure, an uncontrolled loss of proteins is unavoidable.

The tissue fractionation procedure described in this chapter renounces the isolation of defined cell components. This allows us to avoid any selective loss of proteins. Mouse (human) tissues (liver, brain, heart) are fractionated into three fractions:

1. The "supernatant I + II" (SI + II) containing the proteins soluble in buffer (cytoplasmic proteins, nucleoplasmic proteins).

2. The "pellet extract" (PE) containing the proteins soluble in the presence of urea and CHAPS (proteins from membranes and other structures of the cells and cell organelles).
3. The "pellet suspension" (PS) containing proteins released by DNA digestion (histones and other chromosomal proteins).

The SI + II fraction is obtained by homogenization, sonication, and centrifugation of the tissue and the resulting pellet (I), and the combination of the two supernatants gained in this way. The solution thus obtained is the first protein sample. The pellet (II) that remained is extracted with urea and CHAPS, and the homogenate is centrifuged. The supernatant is the PE fraction and gives the second protein sample. The final pellet (III) is suspended into buffer containing benzonase, a DNA-digesting enzyme. The PS (fraction) is the third protein sample. It is applied to 2-DE without further centrifugation.

Care is taken during the whole procedure to avoid any loss of material. In spite of that, some material may become lost, for example, by the transfer of the pulverized, frozen tissue from the mortar to the tube or by removing the glass beads from the sonicated homogenate. However, this does not lead to a preferential loss of certain protein species or protein classes.

It is evident that the best conditions for keeping proteins stable and soluble are given in the living cells *(1,2)*. Therefore, a principle of our tissue extraction procedure was to extract the so-called soluble proteins (SI + II) as far as possible under natural conditions. That means keeping the ionic strength of the tissue homogenate at 150–200 m*M*, the pH in the range of 7.0–7.5, the protein concentration high, and protecting the proteins against water by adding glycerol to the buffer. Generally, the best conditions for the first tissue extraction would be given if the addition of any diluent that disturbs the natural concentration and milieu of the cell proteins were avoided. We prepared a pure cell sap from a tissue by homogenizing the tissue without additives (except for protease inhibitor solutions added in small volumes) followed by high-speed centrifugation, and extracted the pellet that resulted successively in increasing amounts (0.5, 1, or 2 parts) of buffer. The series of protein samples obtained were separated by 2-DE and the patterns compared. The results showed that by increasing the dilution of the proteins, the number of spots and their intensities decreased in the lower part of the 2-DE patterns and increased in the upper part. The same phenomenon, but less pronounced, was observed even when the cell sap was diluted successively. Apparently, low-mol-wt proteins are best dissolved in the pure cell sap and, presumably, tend to precipitate in more diluted extracts. High-mol-wt proteins, in contrast, become better dissolved in more diluted samples. This effect was most pronounced in protein patterns from the liver and not obvious in patterns from heart muscle. This is probably because of the high protein concentration in the liver cell sap that cannot be reached in extracts of other organs.

The dependency of the protein solubility on the molecular weight of the proteins is obscured in 2-DE patterns by another effect that leads to a similar phenomenon. The higher the concentration of the first tissue extract, the higher the activity of the proteases released by breaking the cellular structure by homogenization. Protein patterns from pure liver cell sap, extracted without protease inhibitors (but even with inhibitors) showed an enormous number of spots in the lower part of the gel and a rather depleted pattern in the upper part. In the pH range around 6.0, the protein spots disappeared almost completely, in the upper as well as in the lower part, suggesting that these proteins are most sensitive to the proteases. By extracting the tissue or first pellet with increasing amounts of buffer, the 2-DE pattern (spot number and intensity) shifted from the lower part to the upper part of the gel. Again, this observation was made particularly in liver.

The consequence of these observations for our protein extraction procedure was to gain the supernatant I and II at concentrations that keep all the soluble proteins in solution, but do not reach a level where proteases cannot be inhibited effectively enough. We introduced buffer factors that determine the concentration of the different extracts of each organ. The optimum concentrations were determined experimentally. The optimum was considered to be reached when a maximum of spots occurred in the upper as well as in the lower part of the 2-DE protein pattern. The region around pH 6.0 should not tend to become depleted, starting from the top.

The method described in the following was developed with mouse tissues, but was found to be applicable in the same manner for the corresponding human tissues.

2. Materials
2.1. Equipment

1. Sonicator for performing sonication in a water bath: A small apparatus is preferred (Transsonic 310 from Faust, D-78224 Singen, Germany).
2. Glass beads added to the tissue sample for sonication: The size (diameter) of the glass beads should be 2.0–2.5 mm. The factor 0.034 is calculated for this size of beads (*see* **Note 6**).
3. Mortar and pestle: Form and size of this equipment are shown in **Fig. 1**. Mortar and pestle are manufactured from achat or from glass (WITA, Potsdamer Str. 10, D-14513 Teltow, Germany). Glass was found to be more stable in liquid nitrogen.
4. A small spatula was formed into a shovel by wrought-iron work (**Fig. 1**) and used to transfer tissue powder from the mortar to tubes.

2.2. Reagents

1. Buffer A: The composition is given in **Table 1**. The final solution is filtered, aliquoted into 150 µL portions, and stored at –70°C.
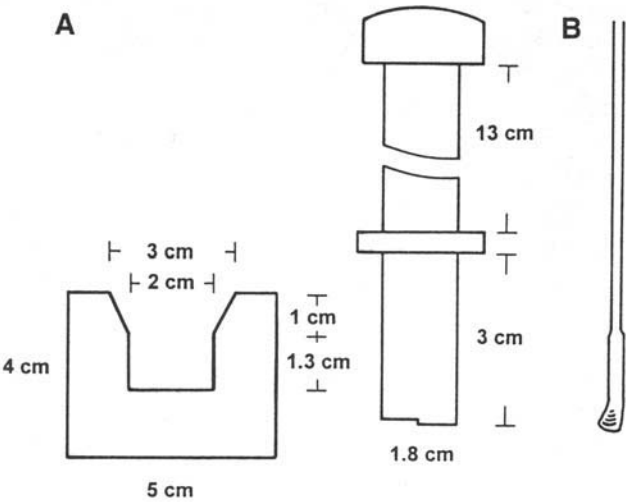
Fig. 1. Special equipment for the pulverization of frozen tissue. (**A**) Glass mortar and plastic pestle. (**B**) Spatula used to transfer the frozen tissue from the mortar to the test tube. A regular spatula was formed to a small shovel.

Table 1
Buffer A

Components	Mixture	Final concentrations
Tris	0.606 g	50 mM
KCl	0.746 g	100 mM
Glycerol	20.000 g	20%
Buffer A	in 100 mL bidistilled water[a]	

[a]pH 7.1 (room temperature), adjusted with HCl.

2. Buffer B: The composition is given in **Table 2**. The final solution was filtered, aliquoted precisely into 900-μL portions, and stored at –70°C. When used, 100 μL of an aqueous CHAPS solution are added. The CHAPS concentration in this aqueous solution is calculated in such a way (*see* **Table 6**) that the pellet II/buffer homogenate (*see* **Table 6**) contains 4.5% CHAPS. This concentration is found to be the best when 2-DE patterns are compared, which resulted from protein samples containing different CHAPS concentrations.
3. Buffer C: The composition is given in **Table 3**. The final solution is filtered, aliquoted into 1-mL portions, and stored at –70°C.
4. Protease inhibitor 1A: one tablet of Complete™ (Boehringer Mannheim, D-68305, Mannheim, Germany) is dissolved in 2 mL buffer A (according to the manufacturer's instructions), and the resulting solution aliquoted into 50-, 80-,

Table 2
Buffer B

Components	Mixture	Final concentrations[a]
KCl	1.491 g	0.2 M
Glycerol	20.000 g	20%
Phosphate buffer[b]	50.000 mL	0.1 M
Buffer B	in 90 mL bidistilled water	

[a]Concentrations in 100 mL of buffer B/CHAPS; *see* **Table 6**.
[b]Phosphate buffer: 33 mL 0.2 M NaH_2PO_4 solution + 67 mL 0.2 M Na_2HPO_4 solution; resulting pH: 7.1.

Table 3
Buffer C

Components	Mixture	Final concentrations
Tris	0.606 g	50 mM
$MgSO_4 \cdot 7H_2O$	0.049 g	a
Buffer C	in 100 mL bidistilled water[b]	

[a]If 1 part (mg) of pellet III is homogenized with 1 part (µL) of buffer C (*see* **Table 6**), the resulting concentration of $MgSO_4$ is 1 mM.
[b]pH 8.0 (room temperature), adjusted with HCl.

Table 4
Protease Inhibitor 2

Components	Stock solutions	Mixture
Pepstatin A	9.603 mg[a] in 100 mL ethanol	10 mL[c]
PMSF	1.742 g[b] in 100 mL ethanol	10 mL
Protease inhibitor 2		20 mL

[a]Concentration in the homogenate (*see* **Table 6**): 1.4 µM.
[b]Concentration in the homogenate (*see* **Table 6**): 1.0 mM.
[c]The solution was incubated in a 37°C water bath to dissolve pepstatin A. The solution should be well-capped.

and 100-µL portions. Inhibitor 1B was prepared in the same way, but with buffer B (900 µL buffer + 100 µL H$_2$O) and aliquoted into 30- and 50-µL portions. Inhibitor 2 is prepared as indicated in **Table 4** and aliquoted into 100-µL portions. The inhibitor solutions are stored at –70°C.

5. DTT-solution: 2.16 g DTT are dissolved in 10 mL bidistilled water. The solution is aliquoted into 100-µL portions and stored at–70°C.
6. Sample diluent: The composition is given in **Table 5**. The solution is aliquoted into 250-µL portions and stored at –70°C.

Table 5
Sample Diluent

Components	Mixture	Final concentrations
Urea	1.08 g (=0.80 mL)	9.000 M
DTT solution[a]	0.10 mL	0.070 M
Servalyt, pH 2.0–4.0[b]	0.10 mL	2.000%
Bidistilled water	1.00 mL	50.000%
Sample diluent	2.00 mL	

[a]*See* **Subheading 2.2, item 5**.
[b]Serva (D-69115, Heidelberg, Germany).

3. Methods

3.1. Extraction of Total Liver Proteins

3.1.1. Dissection of Mouse Liver

1. Kill the mouse by decapitation. Thereby, the body is allowed to bleed. The following steps are performed in the cold room.
2. Cut open the abdomen, cut through the vena femoralis on both sides, and perfuse the liver with 5 mL saline (0.9% NaCl solution).
3. Dissect the complete liver from the body, remove the gallbladder without injury, and cut the liver into its different lobes. The central part of each lobe, i.e., the region where the blood vessels enter the liver lobe, is cut off as well as remainders of other tissues (diaphragm, fascia).
4. Cut the liver lobes into two to four pieces, rinse in ice-cold saline and leave there. Immediately after this step, the next organ is prepared from the same animal, if desirable, and brought to the same stage of preparation as the liver.
5. Cut the liver pieces into smaller pieces (about 5 × 5 mm), and place each piece on filter paper, immerse into liquid nitrogen, and put into a screw-cap tube in which all pieces are collected. For the time of preparation, the tubes are kept in a box containing liquid nitrogen; then they are stored at –70°C.

3.1.2. Extraction of the Liver Proteins Soluble in Buffer (Supernatant I + II)

1. Fill the frozen liver pieces into a small plastic tube of known weight and weigh quickly without thawing. The weight of the liver tissues should be between 240 and 260 mg (*see* **Note 5**).
2. Place a mortar, a pestle, and a small metal spoon into a styrofoam box that contains liquid nitrogen up to a height not exceeding that of the mortar.
3. Put the frozen liver pieces into the mortar, and add buffer A, and inhibitor 1A and 2. The required volumes of each of the solutions are calculated as indicated in **Table 6**. The precise amount of each solution is pipeted as a drop onto the spoon that was kept in the N_2-box. The solution immediately forms an ice bead that can easily be transferred into the mortar.

Table 6
Tissue Protein Extraction Protocol

SI + II fraction: liver			Brain	Heart
Liver pieces		250 mg[b] (A)[c]	240–260 mg fine pieces	100–130 mg (total heart)
Buffer A (liver mg × 1.5)[a]		375 μL	No buffer	↑, Factor 1.0
	$\sum_1$	625		
Inhibitor 1A ($\sum_1$ × 0.08)		50 μL	↑	↑
Inhibitor 2 ($\sum_1$ × 0.02)		12.5 μL	↑	↑
	$\sum_2$	688		
Liver powder			↑	↑
Sonication, 6 × 10 s			No sonication	↑, 12 × 10 s
Number of glass beads ($\sum 2$ × 0.034)		23		
Centrifugation			↑	↑
Supernatant I store frozen			↑	↑
Pellet I weigh		110 mg[d]	↑	↑
Buffer A (pellet I mg × 2)		220 μL	↑ Factor 0.5	↑, Factor 1.0
	$\sum_3$	330		
Inhibitor 1A ($\sum_3$ × 0.08)		26.4 μL	↑	↑
Inhibitor 2 ($\sum_3$ × 0.02)		6.6 μL	↑	↑
Pellet powder			↑	↑
			Sonication, 6 × 10 s	
			Number of glass beads ($\sum_3$ + [$\sum_3$ × 0.08] + [$\sum_3$ × 0.02]) × 0.034	
Stirring			No stirring	Stirring
Centrifugation			↑	↑
Supernatant II add to I			↑	↑
Supernatant I + II weigh		628 mg[e] (B)[c]	↑	↑

73

Table 6 *(continued)*

SI + II fraction: liver		Brain	Heart
Aliquot of supernatant I + II (store rest of supernatant I + II frozen)	50 μL	↑	↑
Urea (50 μL × 1.08)	54 mg	↑	↑
DTT solution (50 μL × 0.1)	5 μL	↑	↑
Ampholyte pH 2–4 (50 μL × 0.1)	5 μL	↑	↑
Final volume of 50 μL supernatant plus additives	400 μL	↑	↑
Diluent	100 μL	No diluent	Diluent
Supernatant I + II, ready for use, store frozen in aliquots	200 μL	↑	↑
2-DE	8 μL/gel	9 μL/gel	6 μL/gel

PE fraction: liver			Brain	Heart
Pellet II weigh		92 mg^d (C)^c	↑	↑
Buffer B/CHAPS (pellet II mg × 1.6)		147 μL	↑, Factor 1.4	↑, Factor 2.2
900 μL buffer B			900 μL buffer B	900 μL buffer B
73 mg CHAPS (displace 69 μL)			77 mg CHAPS	65 mg CHAPS
31 μL bidistilled water			27 μL bidistilled water	38 μL bidistilled water
1000 μL buffer B/CHAPS			1000 μL buffer B/CHAPS	1000 μL buffer B/CHAPS
	$\sum_4$	239		
Inhibitor 1B ($\sum_4$ × 0.08)		19 μL	↑	↑
	$\sum_5$	19 + 147^f = 166		
Pellet powder			↑	↑
Stirring			↑	↑
Urea ([pellet II mg × 0.3] + $\sum_5$) × 1.08		207 mg	↑, Pellet factor 0.56	↑, Pellet factor 0.25
DTT solution			↑, Pellet factor 0.56	↑, Pellet factor 0.25
([Pellet II mg × 0.3) + $\sum_5$]) × 0.1		19 μL		
Stirring			↑	↑

		liver	Brain	Heart
Centrifugation			↑	↑
Supernatant III weigh		375 mg[e] (D)[c]	↑	↑
Ampholyte pH 2–4		20 µL	↑	↑
(supernatant III mg × 0.0526)				
Pellet extract, ready for use,			↑	↑
store frozen in aliquots				
2-DE		8 µL/gel	8 µL/gel	7 µL/gel

PS fraction: liver			Brain	Heart
Pellet III		69 mg[d]	↑	↑
Buffer C (pellet III mg × 1.0)		69 µL	↑	↑
	$\sum_6$	138		
Pellet powder			↑	↑
Benzonase ($\sum_6$ × 0.025)		3.5 µL	↑	↑
Stirring			↑	↑
	$\sum_7$	3.5 + 69[g] = 73		
Urea ($\sum_7$ × 1.08)		79 mg	↑	↑
DTT solution ($\sum_7$ × 0.1)		7.3 µL	↑	↑
Stirring			↑	↑
Ampholyte pH 2.0–4.0			↑, Pellet factor 0.56	↑, Pellet factor 0.25
[(pellet III mg × 0.3) + $\sum_7$] × 0.1		9.4 µL		
Pellet suspension, ready for use,				
store frozen in aliquots				
2-DE		9 µL/gel	8 µL/gel	8 µL/gel

[a]All factors used in this table are explained in **Note 3**.

[b]This figure is given as an example. The amount of the starting material may vary from 240 to 260 mg (*see* **Note 5**).

[c]B ÷ A = control value; D ÷ C = control value (*see* **Note 4**).

[d]This figure is given as an example and is not the result of a calculation. The pellet weight varies because slight losses of material are unavoidable, even during very precise work.

[e]*See* [d] for pellets; this also holds true for supernatants.

[f]Buffer B/CHAPS volume.

[g]Buffer C.

4. Grind all the frozen components in the mortar to powder. Care should be taken that small pieces of the material do not jump out of the mortar when starting to break up the hard frozen material.

5. Transfer the powder into a 2-mL Eppendorf tube using a special spatula (**Fig. 1**). Forceps are used to freeze the tube briefly in N_2 and then to hold the tube near to the mortar in the N_2-box. Care is taken not to leave any powder in the mortar or at the pestle. For collection of this powder, always use the same type of plastic tube. This contributes to the reproducibility of the following sonication step. Compress the powder collected in the tube by knocking the tube against the mortar. The powder can be stored at –70°C or immediately subjected to sonication.

6. For sonication, a calculated number of glass beads (*see* **Table 6** and **Note 6**) is given to the sample powder, and the powder is then thawed and kept in ice. Sonication is performed in a waterbath. The fill height of the water is critical for the sonication effect and should always be at the level indicated by the instruction manual of the apparatus. Furthermore, when dipping the sample tube into the water, it is important to do this at a "sonication center" visible on the concentric water surface motion and noticeable when holding the tube with the fingers into this center. We prefer a small sonication apparatus that forms only one sonication center (*see* **Subheading 2.1.**). The water must be kept ice-cold. Sonication is performed for 10 s. Immediately thereafter, the sample is stirred with a thin wire for 50 s with the tube still being in the ice water. The tube is then kept in ice for 1 min. Then the next sonication round is started, until a total of six 2-min rounds has been reached (*see* **Note 6**). After sonication, the glass beads are caught with fine forceps, cleaned as thoroughly as possible at the inner wall of the vial, and removed. The homogenate sticking on the wall is collected onto the bottom of the tube by a few seconds of spinning. The homogenate is then frozen in liquid nitrogen.

7. Detach the frozen homogenate in the tube from the wall by quickly knocking the top of the tube on the table. Transfer the frozen piece of homogenate into a centrifuge tube (before this, determine the weight of the tube) and thaw. Centrifuge the homogenate at 50,000 rpm (226,000g max.) for 30 min at 4°C.

8. Completely withdraw the supernatant (I) with a Pasteur pipet and fill into a small test tube the dead weight of which has been determined before. The centrifuge tube is kept on ice, and the pipet is put into the tube with the tip at the center of the bottom (the pellet sticks to the wall if a fixed-angle rotor was used). In this position, remainders of the supernatant of the bottom of the tube and inside the pipet accumulate in the pipet, and are added to the test tube. Then the supernatant is frozen in liquid nitrogen and stored at –70°C.

9. Weigh the pellet (I) left in the centrifuge tube on ice, and add buffer A at amounts calculated as indicated in **Table 6**. Mix the pellet and buffer by vortexing, collect the homogenate on the bottom by a short spin, freeze in liquid nitrogen, and store at –70°C or treat further immediately.

10. Grind the homogenate together with inhibitors 1A and 2 to powder as described above for the liver pieces. Transfer the homogenate frozen from the centrifuge

tube to the mortar after detaching the frozen homogenate from the wall by knocking onto the bottom of the tube. Transfer the powder back into the used centrifuge tube, taking care that no powder remains in the mortar.

11. Thaw the powder, and slowly stir the homogenate for 45 min in the cold room.
12. Centrifuge the homogenate as described in **step 7**.
13. Completely withdraw the supernatant (II) from the pellet. This is done in such a way that a white layer, which partially covers the surface of the supernatant, sinks unaffected onto the pellet. Collect the remainders of the supernatant as mentioned in **step 8**. Add supernatant II to supernatant I, and thoroughly mix the two solutions. Measure the weight of the total supernatant.
14. Take a 50-µL aliquot from the total supernatant, and mix with urea, DTT solution, and ampholyte, pH 2.0–4.0, as indicated in **Table 6**. The final concentrations of these components are: 9 M urea, 70 mM DTT, and 2% ampholytes. These three components should be added to the supernatant in the order given here, and each component should be mixed with the supernatant before adding the next one. The final volume of this supernatant mixture is 100 µL. To this, add 100 µL sample diluent (*see* **Table 5**) and mix.
15. The resulting solution is the final sample (supernatant I + II, SI + II). Divide the sample into several portions, freeze each portion in liquid nitrogen, and store at –70°C. As a standard, 8 µL of the sample are applied to the IEF gel, if the large gel 2-DE technique described in Chapter 18 is used (*see* **Note 7**). Freeze the remaining portion of the pure supernatant and store.
16. Determine the weight of the pellet (II). Collect the pellet onto the bottom of the tube by a short spin, then freeze in liquid nitrogen, and store at –70°C.

3.1.3. Extraction of the Pellet Proteins Soluble in the Presence of Urea and CHAPS (Pellet Extract)

1. Grind pellet II, buffer B/CHAPS and inhibitor 1B to powder in a mortar placed in liquid nitrogen (*see* **Subheading 3.1.2.**, **steps 2–4**). The calculation of the buffer and inhibitor volumes is given in **Table 6**. Place the powder back to the centrifuge tube, trying to leave no remainders in the mortar or on the pestle.
2. Thaw the powder mix, and stir slowly for 60 min in the cold room (CHAPS reaction).
3. Add urea (for the amount, *see* **Table 6**) to the homogenate, and stir the mixture for 45 min at room temperature (urea reaction). Some minutes after adding urea, a great part of the urea is dissolved. At this stage, add DTT solution (for the amount, *see* **Table 6**).
4. Remove the magnet rod from the homogenate. At this step, also avoid any loss of homogenate. Centrifuge the homogenate at 17°C for 30 min at 50,000 rpm (226,000g max.).
5. Completely withdraw the supernatant (III) with a Pasteur pipet, and fill into a small test tube the dead weight of which was determined. Collect the remainders of the supernatant as mentioned in **Subheading 3.1.2.**, **step 8**. Measure the weight of the supernatant.
6. Add ampholytes, pH 2.0–4.0 (for the amount, *see* **Table 6**) to the supernatant, and immediately mix with this solution.

7. The resulting solution is the final sample (PE). Divide the sample into several portions, freeze each portion in liquid nitrogen, and store at –70°C. The standard volume of the sample applied per IEF gel is 8 μL, if the large gel 2-DE technique (*see* Chapter 18) is used (*see* **Note 7**).

8. Measure the weight of the pellet (III). Collect the pellet on the bottom of the tube by a short spin, then freeze in liquid nitrogen, and store at –70°C.

3.1.4. Suspension of the Remaining Pellet (Pellet Suspension)

1. Grind pellet III and buffer C to powder in a mortar as described in **Subheading 3.1.2.**, **steps 2–4**. The buffer volume is calculated as indicated in **Table 6**. The powder is transferred into a test tube, thereby avoiding any loss of material.

2. Thaw the powder, and add benzonase (for the amount, *see* **Table 6**) in the form offered by the manufacturers (Merck, D-64271, Darmstadt, Germany). Slowly stir the homogenate for 30 min in the cold room (DNA digestion).

3. Add urea (for the amount, *see* **Table 6**), and stir the homogenate at room temperature for another 30 min. During this time, add DTT solution (for the amount, *see* **Table 6**) once the major part of urea is dissolved. At the end of this period, add ampholytes, pH 2.0–4.0 (for the amount, *see* **Table 6**), and quickly mix with the homogenate.

4. The resulting solution is the final sample (PS). The sample is frozen in liquid nitrogen and stored at –70°C. The standard volume of the sample applied per gel (large gel 2-DE; *see* Chapter 18) is 9 μL (*see* **Note 7**). The sample contains some fine, unsolved material and is therefore transferred to the gel with a thin Pasteur pipet instead of a microliter syringe.

3.2. Extraction of Total Brain Proteins

3.2.1. Dissection of Mouse Brain

1. Kill the mouse by decapitation. The following steps are performed in the cold room. If several organs have to be taken from the same animal, start with the brain.

2. Cut off the skin of the head, and open the cranium starting from the spinal canal proceeding in frontal direction. Break the cranial bones apart so that the brain is exposed. Take out the brain, including the two bulbi olfactorii and a short piece of the spinal cord. Place the brain into a Petri dish containing ice-cold saline. Remove any blood vessels and blood at the outside of the brain.

3. Cut the brain into four pieces, place the pieces on filter paper, and then individually immerse into liquid nitrogen and collect in a screw-cap tube. Keep the tubes in liquid nitrogen and finally store at –70°C.

3.2.2. Extraction of the Brain Proteins

The four pieces of a brain are put frozen into a mortar that was placed into a box containing liquid nitrogen and crushed with the pestle to fine pieces. The crushed material is transferred completely back to two test tubes in such a way that one of these tubes contains 240–260 mg of the frozen tissue (weigh the tube without thawing the tissue).

The weighed material is used to prepare the supernatant I + II, the pellet extract, and the pellet suspension. The procedure follows that of liver extraction with some exceptions, which are indicated in **Table 6**. One exception is that the tissue powder is produced without buffer and subjected to centrifugation without sonication. (A rather small amount of supernatant results.) Sonication is performed with the pellet I homogenate.

3.3. Extraction of Total Heart Proteins

3.3.1. Dissection of Mouse Heart

1. Kill the mouse by decapitation. The following steps are performed in the cold room.
2. Open the thorax, and remove the heart. Place the heart into a Petri dish containing ice-cold saline. Cut off the two atria, and open the ventriculi to remove any blood and blood clots.
3. Dry the heart on filter paper, freeze in liquid nitrogen, and store in a screw-cap tube at –70°C.

3.3.2. Extraction of the Heart Proteins

The supernatant I + II, the pellet extract, and the pellet suspension are prepared from a single heart. The procedure is as described for liver with some modifications. The modifications are indicated in **Table 6**.

4. Notes

1. Maximum resolution of tissue protein fractions by 2-DE: **Fig. 2** shows the 2-DE patterns of the three protein fractions SI + II, PE, and PS of the mouse liver. The SI + II fraction reveals the highest number of protein spots. When the spots were counted visually, i.e., by placing the 2-DE gel on a light box and dotting each spot with a pencil *(3)*, about 9200 proteins were detected in this fraction. The SI + II pattern of the brain revealed about 7700 proteins, that of the heart being about 4800 protein spots. The high spot numbers reflect the high resolution of the large gel 2-DE (*see* Chapter 18), which reveals many weak spots between the major spots. All these spots were counted precisely. The analysis of the large gel patterns by laser densitometry results in 24.5 million data. Treatment of the data by special computer programs for spot detection and correction of the computer pattern against the original gel pattern led us to the maximum number of spots we could obtain. For example, the SI + II pattern of brain evaluated in this way revealed about 8500 protein spots compared to 7700 spots detected by visual inspection of this pattern.
2. Effect of fractionated extraction of tissue proteins: The purpose of fractionating the proteins of a certain tissue was to increase the number of proteins detectable in this tissue by 2-DE. This purpose, however, would only be fulfilled if each fraction contained a notable number of proteins that are strongly fraction-specific, so that the total tissue proteins can be distributed over several gels. Comparison of the 2-DE patterns from the SI + II and PE fractions of the liver (**Fig. 2**)
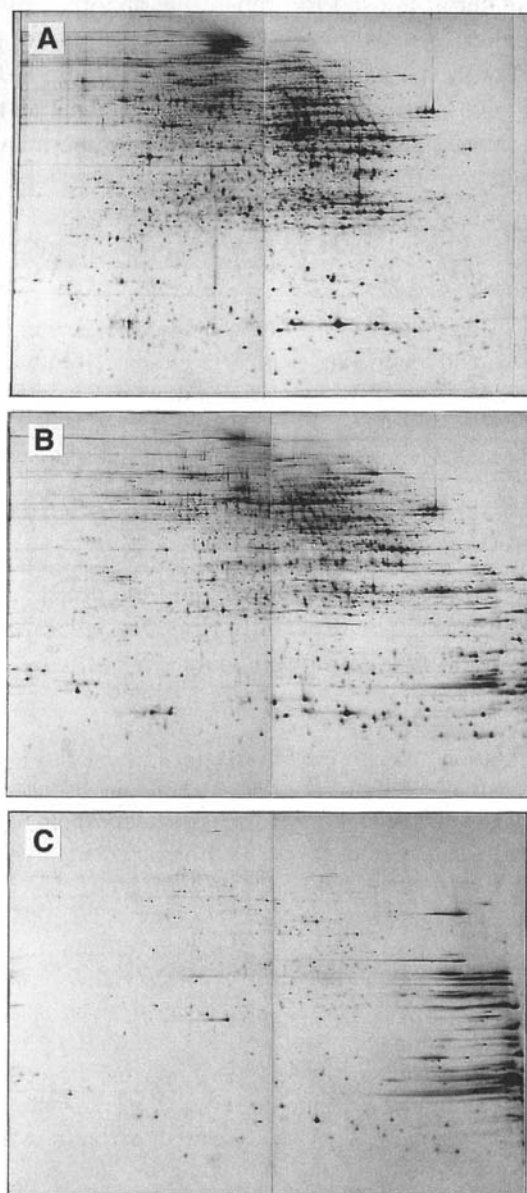
Fig. 2. 2-DE protein patterns from mouse liver. The tissue was fractionated into supernatant I + II **(A)**, pellet extract **(B)**, and pellet suspension **(C)** as described in **Subheading 3.** The three fractions were subjected to the large gel 2-DE (*see* Chapter 18). In the pellet extract pattern, many protein spots occur that are not present in the supernatant pattern and vice versa. Pellet-specific spots occur more in the basic half, and supernatant-specific spots more in the acid half of the pattern. The pellet suspension pattern

showed that the PE pattern revealed about 2000 protein spots not detectable among the 9200 spots of the SI + II pattern. The PS pattern revealed only about 70 additional spots. The PS protein spots represent the class of the most basic proteins (**Fig. 2**) and belong mainly to the chromosomal proteins (e.g., histones). Therefore, the PS fraction is only of interest when the class of very basic proteins is subject of the investigation.

Considering the 2-DE patterns of the SI + II and PE fraction in more detailed (**Fig. 3**), quite a number of very prominent spots can be observed that occur in one pattern, but do not occur, even in trace amounts, in the other pattern. At the same time, other spots revealing only low intensities are present in both patterns. This suggests that contamination of one fraction by the other one scarcely affects the fraction specificity of the SI + II and PE pattern, but, apparently, many proteins exist naturally in both the cytoplasm and the structural components of the cell.

Taking all three fractions into account, the total liver proteins could be resolved into about 11,270 different proteins (polypeptide spots). This, however, does not mean that the protein sample preparation procedure described here—in combination with the 2-DE technique described in Chapter 18—revealed all the different proteins of the liver. Many proteins may exist in a tissue in undetectable amounts, and special cell fraction procedures followed by protein concentration steps would be required to detect these proteins. We isolated cell nuclei from liver and brain, and extracted the nuclear pellet in a similar way to that for the tissues. The 2-DE patterns showed that the nuclear extracts add a large number of new proteins to those already known from the tissue extract patterns. However, protein spots present in both the nuclear and the tissue extracts occur as well, particularly in the acid halves of the supernatant patterns. In general, the proteins represented by the SI + II, PE, and PS patterns can be considered as the main population of protein species of a tissue to which further species can be added by analyzing purified and concentrated subfractions.

3. Explanation of factors used in **Table 6**: Factors were calculated to determine the amounts of urea, DTT solution, and ampholytes necessary to transmute any volume of a solution (theoretically water) into a mixture containing 9 *M* urea, 70 m*M* DTT, and 2% ampholytes. Calculations of the factors: 500 μL water (aqueous protein extract) + 540 mg urea (displaces 400 μL) + 50 μL DTT solution (*see* **Subheading 2.2., step 5**) + 50 μL ampholyte solution (commercial solutions that usually contain 40% ampholytes) = 1000 μL. If the volume of a protein solution to be mixed with urea, DTT, and ampholytes is n μL, the amounts of the components to be added are: $(540 \div 500) \times n = 1.08 \times n$ mg urea, $(500 \div 50) \times n = 0.1 \times n$ μL DTT solution and $0.1 \times n$ μL ampholyte solution.

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reveals the very basic proteins of the tissue extracts. Because the IEF gels do not cover the entire basic pH range, these proteins cannot reach their isoelectric points. To prevent these proteins from accumulating at the end of the gel, the IEF run was shortened by 2 h at 1000 V. Consequently, the very basic proteins form streaks instead of focused spots. For the evaluation of the patterns in terms of number of spots, *see* **Notes 1** and **2**.
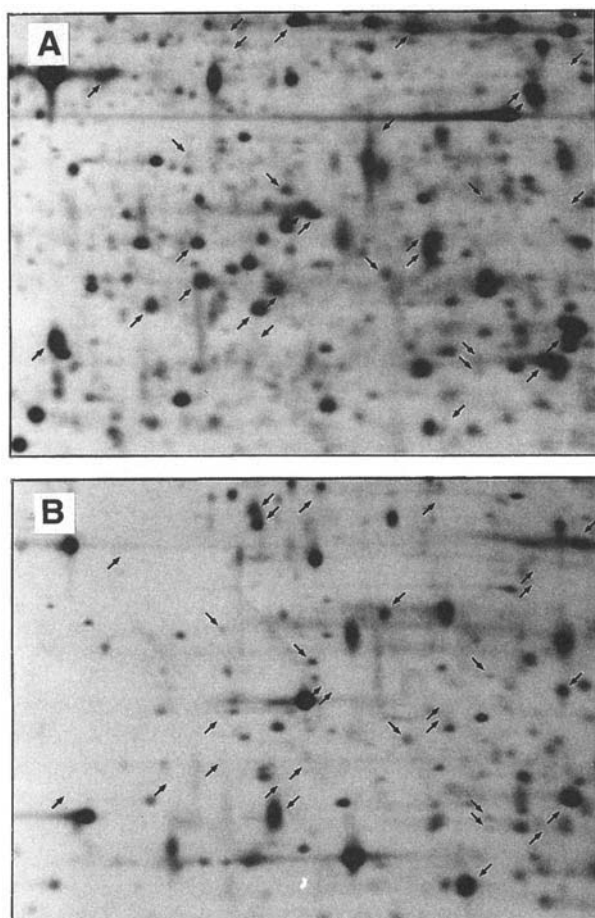
Fig. 3. Sections from 2-DE patterns shown in **Fig. 2**. The supernatant I + II **(A)** and the pellet extract **(B)** of the liver are compared. Some of the prominent protein spots present in the supernatant pattern (↗) but completely absent in the pellet extract pattern (↗), the reverse situation (↙), and some spots of low intensity present in both patterns (↘) are indicated. Other spots show high intensity in one pattern, but low intensity in the other one. These spots may reflect naturally occurring unequal distributions of proteins between the two different fractions rather than contaminations of one fraction by the other one (*see* **Note 2**).

If the protein solution already contains urea and DTT (*see* **Table 6**: PE preparation), the factor 0.0526 is used to calculate the ampholyte volume for this solution. Calculation of the factor: (500 μL extract + 400 μL urea + 50 μL DTT solution) ÷ 50 μL ampholyte solution = 0.0526.

If the protein solution of *n* μL includes a cell pellet, i.e., insoluble material, the *n* μL volume should be reduced by the volume of the insoluble material (theoreti-

cally by the volume of the dry mass of this material). For this reason, the pellet factor (e.g., 0.3) was introduced. This factor was determined experimentally using urea as an indicator. The factor reduces the volume n μL of a protein solution (containing a pellet) to the volume n' μL; n' μL × 1.08 results in an amount of urea that is added to the n μL of the solution, at the border of solubility, i.e., about 9 M. Note that pellet III contains urea and DTT by the foregoing steps. Therefore, in this case, the pellet volume was not taken into account when calculating the amounts for urea and DTT to be added to the final pellet suspension. In all these calculations, no distinction was made between values measured in volumes (μL) and values measured in weights (mg). This makes the calculation somewhat incorrect, but practicable and more reproducible.

The inhibitor 2 solution was prepared as concentrated as possible to keep the volume of this solution small ($\frac{1}{50}$th of the homogenate volume, i.e., factor 0.02). This allowed us to ignore the error that resulted when the inhibitor solution was added to instead of included in the volume of the homogenate. The factor 0.08 for the inhibitor 1 solution was derived from the instructions of the manufacturers of the Complete™ tablets: 1 tablet should be dissolved in 2 mL buffer and this volume added to 25 mL of the homogenate, i.e., the volume of the inhibitor 1 solution to be added to n μL homogenate is $(2 \div 25) \times n = 0.08\ n$ μL.

The volume of benzonase solution (ready-made solution of the manufacturer) necessary to digest the DNA in the pellet III suspension was determined experimentally. A chromatin pellet was prepared from isolated liver cell nuclei and found to change from a gelatinous clot to a fluid if treated as follows: 1 g chromatin pellet + 1 mL buffer + 0.050 mL benzonase solution (= 0.025 mL Benzonase/1 mL homogenate), stirred for 30 min at 4°C.

The buffer factors used to calculate the volumes of the buffer added to the tissues or pellets are explained in the **Subheading 1**. Note that the tissue to be homogenized must be free of any wash solutions. Otherwise, the calculated buffer volume becomes falsified.

4. Control values: Control values were calculated for each sample prepared by the fractionated extraction procedure to monitor the correctness and reproducibility of the preparation. The calculation of the control values is indicated in **Table 6**. In the following, an example is given from a real experiment. From a series of 73 individual mouse hearts, the SI + II fractions were prepared and the control values B ÷ A (*see* **Table 6**) were calculated: 60 samples showed values between 1.97 and 2.10, three samples between 1.94 and 1.96, and six samples between 2.11 and 2.13. Four samples with greater deviations (1.90, 1.91, 2.16, 2.24) were excluded from the investigation. The range 1.97 and 2.10 (mean 2.04 ± 0.04) was taken as standard control value for the preparation of mouse heart SI + II samples.

5. Amount of tissue used as starting material: The amount of tissues given in **Table 6** can be increased, but should not exceed 500 mg. However, the tissue amounts indicated give enough sample solutions to run many 2-DE gels, so that less rather than more material can be used. In cases in which only very small amounts of tissue are available (e.g., 2–5 mg heart biopsy samples, 10–12 mg of two mouse

eye lenses, early mouse embryos), a total protein extract is prepared instead of SI + II, PE, and PS fractions. The tissue is pulverized with buffer B/CHAPS (the buffer factor is less critical here than in fractionated extraction and should not be two low) and inhibitors 1B and 2, sonicated, stirred for 30 min at 4°C, weighed, mixed with urea, DTT solution, and ampholyte solution (*see* **Note 3**), stirred for 45 min at room temperature, and used without centrifugation. Small plastic tubes and a glass rod with a rough surface at the well-fitting tip may serve as mortar and pestle. Total protein extraction was also preferred when a 2-DE pattern of low complexity was expected, for example, protein patterns from cultured human fibroblasts (*see* Chapter 18, Fig. 6; buffer factor 1.25 was used in this case).

6. Sonication: The conditions for sonicating mouse tissue homogenates (liver, brain, heart) were determined with the aim of breaking the membranes of all the cells and cell nuclei of the tissue. Three parameters were varied in the experiments: the length of the period of sonication, the number of repeats of sonication, and the number of glass beads added per volume of the homogenate. The effect of the various conditions was checked under the microscope by inspection of the sonicated material. During 10 s of sonication, the temperature of the homogenate increased from 0°C to 11–12°C. Therefore, sonication was not performed for more than 10 s. Glass beads are essential for breaking the cellular structures (membranes).

   Since most of the homogenates are rather thick fluids, the beads cannot flow freely. Consequently, for a given volume of homogenate, a certain number of glass beads is necessary to expose the homogenate evenly to the sonication effect. This number was standardized by using the factor 0.034 in calculating the number of glass beads for a given volume of homogenate.

   It follows from the above-mentioned three experimental parameters that the only parameter that can be varied to manipulate the effect of sonication was the number of repeats of the 10-s sonication period. Under the conditions described in the **Subheading 3.**, the membranes of all cells were broken and no longer visible under the microscope. However, a certain number of intact nuclei were still detectable. We did not try to break even these nuclei by extending sonication. Sonication by using a metal tip cannot be recommended. We observed heavily disturbed 2-DE patterns as a result of employing this technique: many protein spots disappeared depending on the extent of sonication, and new spot series occurred in the upper part of the gel, apparently as a result of aggregation of protein fragments.

7. Amount of protein applied per gel: The protein samples applied to the IEF gels (*see* **Table 6**), contain about 100 μg protein. There is, however, no need to determine the protein concentration of each sample prepared in order to obtain protein patterns of reproducible intensities. The concept of the procedure described here for extracting tissues was to keep the volume of the extracts in strong correlation to the amount of the starting material (tissue or pellet) that was extracted. Therefore, by working precisely, the final sample should always contain nearly the same protein concentration. Accordingly, the reproducibility of the pattern inten-

sity depends on the precise sample volume applied to the gel—and, of course, on the protein staining procedure.

The sample volumes per gel given in **Table 6** are adapted for silver-staining protocols. A general guideline may be: decreasing the protein amount per gel and increasing the staining period is better than the other way around; diluted samples at a reasonable volume are better than concentrated protein samples at a small volume. But dilute, if necessary, the final sample (*see* **Table 5**), not the starting material.

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10

Preparation and Solubilization of Body Fluids for 2-D

Jean-Charles Sanchez and Denis F. Hochstrasser

1. Introduction

Cells, tissues, and organs require energy to function normally. Some of them are metabolic providers, and some others are users. Body fluids act as transporters and distributors of metabolic "fuels" between cells and maintain relatively constant conditions through homeostasis. The ratio of solute to water in all body fluids is maintained within a very narrow range. The concentrations of body fluid metabolites are thus extremely well regulated. Many diseases or environment disturbances lead to changes in the expression of body fluid proteins. Information on these changes is often of diagnostic, prognostic, or therapeutic importance.

Recent improvements in two-dimensional gel electrophoresis (2-D PAGE) techniques have facilitated the analysis of the protein content of body fluids. Tracy and Young et al. *(1)* in the early 1980s, and more recently Hochstrasser and Tissot *(2)* demonstrated that 2-D PAGE separation of body fluids may be used as a complementary tool to other clinical chemistry methods to solve several biomedical problems.

The preparation and solubilization of body fluid samples for 2-D PAGE should follow three important rules:

1. As many proteins as possible have to be solubilized.
2. Protein aggregates have to be disrupted, to avoid spot streaking.
3. Sample preparation has to be reproducible in order to avoid misleading results.

To achieve the above rules and obtain an effective 2-D PAGE separation of individual polypeptides, an effective solubilizing buffer generally includes reducing agents, urea, and detergents (nonionic, anionic, cationic, and zwitterionic).

Plasma proteins are major components of most types of body fluids, which themselves are mainly composed of soluble proteins. Some of the body fluids, including serum/plasma or ascitic fluid, have a high protein concentration (40–80 g/L) and do not need any treatment prior to protein solubilization. In contrast, cerebrospinal fluid (CSF) samples have a low protein content and need concentration, whereas urine samples have a high salt concentration and need to be desalted. This chapter describes the preparation and solubilization of the most widely studied body fluids, i.e., serum/plasma, CSF, and urine. Protocols for other body fluids are similar to the methods describe in this chapter. In the following references, specific details can be found for collecting and processing other body fluid including saliva *(3)*, joint fluid *(4)*, tears *(5)*, pancreatic fluid *(6,7)*, amniotic fluid *(8)*, ascitic fluid *(9)*, pleural fluid *(10)*, cyst fluid *(11)*, sweat *(12)*, milk *(13)*, and seminal fluid *(14)*.

2. Materials

2.1. Reagents

1. Buffer A: 10% SDS (w/v), 2.3% DTT (w/v). Store at –70°C in 100-μL aliquots (*see* **Note 1**).
2. ISO buffer: 9 *M* urea, 4% CHAPS (w/v), 35 m*M* Tris, 65 m*M* DTT, and a trace of bromophenol blue. Store at –70°C in 500-μL aliquots (*see* **Note 2**).
3. Ice-cold acetone (stored at –20°C).
4. Sodium azide solution: 5 mg of sodium azide dissolved in 100 mL of Milli-Q H_2O.
5. Microcon-10® microconcentrator (Amicon).

3. Methods

3.1. Plasma and Serum

Anderson and Anderson *(15)* undertook the first 2-D PAGE analysis of plasma proteins using Coomassie blue to stain the proteins. More recently, a silver-stained 2-D PAGE reference map has been established by Golaz et al. *(16)* and is available on the ExPASy molecular-biology server (http://www.expasy.ch/cgi-bin/map2/def?PLASMA).

3.1.1. Plasma Collection and Preparation

1. Collect venipuncture blood in a syringe or tube containing a suitable anticoagulant, such as heparin or EDTA. Capillary blood from a finger prick can be used provided that the skin is first cleaned thoroughly to avoid contamination with sweat.
2. After blood collection, the syringe or tube should immediately be placed in an ice bath and brought to the laboratory for analysis (*see* **Note 3**).
3. On arrival in the laboratory, the blood specimen must be centrifuged immediately at 2000*g* for 10 min at 5°C to avoid hemolysis, and decanted (*see* **Note 4**).
4. The sample can be either processed immediately or stored at –70°C until analysis.

3.1.2. Serum Collection and Preparation

1. Collect venipuncture blood in a sterile tube.
2. After blood collection, the tube should immediately be brought to the laboratory for analysis (*see* **Note 3**).
3. On arrival in the laboratory, allow the blood specimen to clot for 30 min at room temperature. It must then be centrifuged at 2000*g* for 10 min and decanted (*see* **Note 4**).
4. The sample can be either processed immediately or stored at –70°C until analysis.

3.1.3. Plasma/Serum Solubilization

1. Mix an aliquot of 6.3 μL of human plasma/serum with 10 μL of buffer A.
2. Heat the sample to 95°C for 5 min, and then dilute to 500 μL with ISO buffer (*see* **Note 5**). Mix by vortexing.
3. Load 60 μL (45 μg) of the final diluted plasma/serum sample on the first-dimensional separation (*see* **Notes 8**).
4. The remaining sample can be stored for further analysis at –70°C (*see* **Note 6**).

3.2. Cerebrospinal Fluid (CSF)

A "normal" CSF 2-D PAGE protein profile is very similar to the plasma/serum pattern. There are only five major differences:

1. CSF protein content is low, in the range 0.2–0.4 g/L.
2. CSF contains lower amounts of haptoglobin.
3. Apolipoproteins CII and CIII are not detectable.
4. Transthyretin has a higher concentration.
5. Transferrin posttranslational modifications are different.

The CSF 2-D PAGE profile was first studied by Switzer et al. *(17)* and then by Goldman et al. *(18)*.

1. Collect CSF by lumbar puncture in a sterile container. It should immediately be placed in an ice bath and brought to the laboratory for analysis.
2. On arrival in the laboratory, the CSF specimen must be centrifuged to remove circulating cells at 2000*g* for 10 min at 5°C (*see* **Note 4**).
3. The sample can be either processed immediately or stored at –70°C until analysis.
4. Mix an aliquot of 300 μL of human CSF with 600 μL of ice-cold acetone and centrifuge at 10,000*g* at 4°C for 10 min (*see* **Note 7**). Discard the supernatant.
5. Mix the pellet with 10 μL of buffer A.
6. Heat the sample to 95°C for 5 min, and then dilute to 60 μL with ISO buffer (*see* **Note 5**). Mix by vortexing.
7. Load the whole final diluted CSF sample (45 μg) on the first dimensional separation (*see* **Note 8**).

3.3. Urine

The major interest in the analysis of urine in 2-D PAGE is to discriminate between glomerular and tubular diseases. In glomerular diseases, additional

high-mol-wt plasma proteins may be detected in the urine owing to alteration of the glomerus. In contrast, tubular diseases show only additional low-mol-wt proteins. Urine sample preparation, solubilization, and 2-D PAGE maps have been described by Anderson et al. *(19)* and Edwards et al. *(20)*. Urine contains proteins in trace amounts (100 mg/L) and thus needs to be concentrated with concomitant salt removal prior to 2-D PAGE analysis.

1. There are three types of urine collection methods:
   a. Morning specimen.
   b. Random spot specimen.
   c. 24-h Specimen.
      Any of these specimens can be collected in a sterile container and then centrifuged at 2000*g* for 10 min to remove cells, casts and crystals (*see* **Note 4**).
2. In order to inhibit bacterial growth, add 0.1 mg of sodium azide/L of urine.
3. The sample can be either processed immediately or stored at –70°C.
4. Pipet 500 µL of urine into the sample reservoir of a Microcon-10® microconcentrator (*see* **Note 7**).
5. Centrifuge at 14000*g* for 30 min.
6. Add 490 µL of Milli-Q water to the retentate, and centrifuge again at 14,000*g* for 30 min.
7. Remove assembly from centrifuge, place sample reservoir upside down in a new tube, and centrifuge at 1000*g* for 1 min.
8. Between 10 and 20 µL of concentrated and desalted urine will be collected.
9. Mix an aliquot of 10 µL of concentrated urine with 10 µL of buffer A.
10. Heat the sample to 95°C for 5 min, and dilute it to 60 µL with ISO buffer (*see* **Note 5**).
11. Load the whole final diluted urine sample (45 µg) on the first-dimensional separation (*see* **Note 8**).

4. Notes

1. It is important to work with highest-quality reagents, which have been tested for electrophoresis. Commercial bulk chemicals may contain impurities. A typical example is SDS that can be sold as a mixture of different chain lengths, including 12, 14, and 16 carbon chains. DTT has to be freshly prepared and kept absolutely dry. Its oxidation may give rise to spot streaking on the 2-D PAGE. SDS solubilizes protein complexes to their component. It also helps proteins to enter into the IEF gel. It binds most of the proteins in a ratio of 1.4 g SDS/g of proteins *(21)*. It has been shown that the absence of reducing agent in the solubilizing solution may decrease the SDS:polypeptides binding ratio *(21)*. The reduction and alkylation of disulfide bonds is thus important to avoid conformational effects and increase protein solubilization. However, if the SDS:proteins ratio exceeds 3:1, proteins may not migrate properly at the acidic end of the IEF gel.
2. In body fluids, the addition of positively charged ampholytes to prevent sample viscosity from cellular DNA is not necessary.

3. Protein degradation may occur between sample collection and solubilization owing to the presence of proteases. Protease inhibitors, such as PMSF (0.2 m*M*), leupeptine (50 µg/mL), benzamidine (0.8 m*M*), or a combination of these can be added to the sample.

4. Centrifugation is always recommended before sample preparation and solubilization for removal of circulating cells (2000*g* for 10 min at 5°C).

5. Do not heat any solution containing urea above 37°C. Carbamylation of proteins will occur owing to the creation of isocyanates.

6. Samples should be divided into several small aliquots before storage in order to avoid repeated freezing and thawing of a single sample. Solubilized samples can be frozen at –70°C or lower temperatures, and kept for several years. The freezing step should be fast to avoid urea crystal formation. Avoid freezing solubilized samples at –20°C.

7. Low protein abundancy may be overcome using methods that concentrate samples. These include lyophilization, ultrafiltration, and precipitation with, for example, TCA, ammonium persulfate, or ice-cold acetone. If the sample contains a high salt concentration, desalting through dialysis, microfiltration, or gel chromatography will be necessary prior to concentration.

8. In order to shorten the focusing time, increase the loading capacity, and enhance the resolution of 2-D PAGE, the entire IPG gel can be used for sample application, with the proteins entering the gel during rehydration (*see* Chapters 24 and 24a). This method avoids the use of sample cups, eliminates precipitation at the sample application point, and thus improves resolution throughout the pH range of the gel. It also allows precise control of protein amounts and sample volumes loaded into the IPG gels. Similar protocols to those described in this chapter can be used for protein preparation and solubilization. The use of commercial NL 3.5-10 IPG (180 × 3 mm) was optimized for body fluids. It was found that a rehydration volume of 500 µL produced the maximum number of detectable proteins. The in-gel sample rehydrated IPG strips can be frozen at –70°C or lower temperatures until analysis. The strips may also be frozen between first-dimensional and 2-D separation.

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11

2-D Electrophoresis of Plant Proteins

Akira Tsugita and Masaharu Kamo

1. Introduction

The isolation of proteins from plants for two-dimentional electrophoresis (2-DE) is complicated by the plants' hard cell walls, high frequency of modified N-termini, and insolubility of membrane proteins. Some of these difficulties are not limited to plant proteins, but are also rather common for proteins from other organisms.

Recently, we worked on the 2-DE of two plants, *Arabidopsis* (dicot plant) and rice (monocot plant), whose genome projects are much advanced. We used five tissues for *Arabidopsis*, and nine tissues and one organelle for rice. They were edited by comigration with external standardizing proteins *(1)*, as in spot-wise *(2)* or as in cross-stripe-wise *(3)*. 2-DE gels (22.5 × 22.5 cm) are able to separate between 200 and 2000 proteins. Arabidopsis proteins were separated into 4763 protein spots, and 136 proteins were sequenced and 57 proteins were blocked at the N-termini *(4)*. Rice proteins were separated to 4892 protein spots, 176 proteins were sequenced, and 105 proteins were blocked *(2)*.

The number of proteins separated for these plants cover one-forth to one-tenth of the theoretical ones from genomic information. To overcome this discrepancy, we must try to enlarge the 2-DE size and to identify the extremely small-copy-number proteins that have to be isolated from the large copy number proteins.

2. Materials
2.1. Equipment

A mortar with a pestle is needed.

2.2. Reagents

1. TCA solution A: 10% trichloroacetic acid in acetone containing 0.07% 2-mercaptoethanol.

2. Solution B: 0.07% 2-mercaptoethanol in acetone containing 1 mM PMSF and 2 mM EDTA.
3. Liquid nitrogen.
4. IEF and NEpHGE electrofocusing solvent: 9.9 M urea, 2% carrier ampholytes (pH 3.0–10.0, Millipore, Bedford, MA), 100 mM DTT, 3.8% Triton X-100, and 0.03% bromophenol blue *(2)*.
5. IPG electrofocusing sample solvent: 9.0 M urea, 2% carrier ampholytes (Pharmacia, Uppsala, Sweden), 60 mM DTT, 0.5% Triton X-100, and 0.003% bromphenol blue.

3. Methods

3.1. Preparation of Plant Tissue Protein (see Note 1)

1. Store a mortar with a pestle below –20°C, and keep at low temperature.
2. Freeze tissue (e.g., Arabidopsis leaf) at –80°C or lower.
3. While still frozen, cut the tissue into small pieces using scissors.
4. Grind the tissue into a powder using the mortar with a pestle in a 4°C cold room. Add liquid nitrogen to freeze the samples, and keep in the frozen state.
5. Suspend the powder in 10X vol of TCA solution A (*see* **Note 2**).
6. After standing for 45 min at –20°C, centrifuge the suspension at 35,000g for 15 min.
7. Resuspend the precipitates in 10X vol of solution B, and place at –20°C for 1 h.
8. Centrifuge the suspension at 35,000g for 15 min, and then resuspend the precipitates with solution B. Immediately centrifuge the suspension at 35,000g for 15 min.
9. Lyophilize the precipitate for 1 h.
10. Suspend 5 mg of the lyophilized sample powder in 200 μL of the electrofocusing solvent. Incubate the solution at 37°C for 1 h with continuous by stirring and then centrifuged at 19,000g for 10 min.
11. Quantify of the concentration of protein in sample solution as described in **Subheading 3.2.**
12. Add external protein standards *(2,3)* to the supernatant and incubated at 37°C for 30 min (*see* Chapter 30).
13. Subject the supernatants to IEF, NEpHGE, or concentrated using a TCA precipitation (*see* **Note 3**).
14. To concentrate the proteins, add TCA to the supernatant to a final concentration of 20% and incubate on ice for 1 h.
15. Centrifuge the suspension at 19,000g for 10 min, and repeatedly extract the precipitate with diethyl ether.
16. Lyophilize the precipitates, resuspend the powder in 50 μL of the electrofocusing solvent, and quantitate the protein concentration as described in **Subheading 3.2.**
17. Subject the resuspended proteins to 2-DE electrophoresis.

3.2. Quantification of Sample Protein

1. Quantification of the concentration of proteins sample was carried out in the electrofocusing solvent using ovalbumin as a protein standard *(5)* (*see* Chapter 2).

2. Adjust the protein concentration of the sample solution to 1.5–2.0 mg/mL with the electrofocusing solvent.
3. Use 50 μL of the sample for silver-stained 2-DE gels.

4. Notes

1. This preparation method was originally reported by of Darmeval et al. *(6)* with several modifications. Plant cells have a hard cell wall that must be crushed at the start of the preparation.
2. Any protease inhibitor or combinations of inhibitors were found to be efficient enough to stop the protease activities in the sample preparation process. This fact is easily checked by the fragmentation of ribulose *bis*-phoshate caraboxylase (Rubisco) protein on a 2-DE gel *(6)*. In the present method, we employ TCA-acetone treatment in addition to PMSF where acid and organic solvent denature almost all proteins especially proteases. This method is also efficient in the other system in our experience.
3. The TCA-acetone system precipitates proteins together with cell wall and fiber components. These insoluble components are removed when the precipitates are dissolved in electrofocusing buffer and centrifuged.
4. The highly abundant plant proteins, resulting in the large spots may hide low-copy-number proteins in the 2-DE gel. In these cases, prior purification, such as affinity chromatography, is needed to remove the highly abundant proteins.

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12

Quantifying Protein
in 2-D PAGE Solubilization Buffers

Louis S. Ramagli

1. Introduction

Accurate quantitative and qualitative comparison of resolved proteins by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) requires that identical amounts of sample be loaded on each gel. Use of trichloroacetic acid (TCA) or organic solvent precipitation protocols followed by a protein assay results in either over- or underestimation of solubilized protein concentration. Furthermore, the practice of loading equivalent amounts of radioactively labeled protein-based counts/min or loading extract from the same number of cells is inaccurate. The author has observed that radioactively labeled cell extracts exhibit 10–20% variances in protein concentration. Similarly, solubilization of 2×10^6 cells from a number of colorectal carcinoma cell lines resulted in protein concentrations ranging from 1.60 to 3.93 µg protein/µL buffer *(1)*. Therefore, the ability to quantify protein actually solubilized in 2-D PAGE sample buffers is necessary to allow users to adjust for variable solubility properties of protein(s).

In 2-D PAGE solubilization buffers, high concentrations of urea, thiol-reducing agents, nonionic detergent, such as NP40, and in some cases, sodium dodecyl sulfate (SDS) or salts are required for disruption of aggregates and complexes of protein into pools of individual polypeptides. All of these reagents interfere with protein assays to some degree. Although many solutions have been suggested for each reagent individually *(2–6)*, prior to development of the method described here *(1)*, no method had dealt with a mixture of necessary buffer components.

Protein concentration in complex 2-D PAGE buffers cannot be directly determined by the Lowry, Biruet, BCA, or Bradford methods owing to one or

more interfering compounds *(7–9)*. The Lowry, Biruet, and BCA methods are all based on the reduction of Cu^{2+} to Cu^+ for development of color *(9)*. Reducing agents, such as thiol compounds, interfere with these assays *(2)*. Although the Bradford agent is only mildly affected by these compounds, the binding of Coomassie G-250 dye to proteins is affected by basic reagents, such as urea, and basic carrier ampholytes. The modified Bradford protein assay described here takes advantage of the Bradford assay's insensitivity to many of the reagents in these mixtures while correcting for the affects caused by basic reagents. The use of dilute acid to neutralize the sample prior to adding the dye produces a biphasic response over the concentration range of 0.5–50 µg *(1)*. The nonlinearity over this entire range is inherent to all protein assays using Coomassie brilliant blue G-250 and thought to be caused by overlap in respective spectrums of the two-color forms of the dye (*see* **Note 8**).

2. Materials

1. Coomassie brilliant blue G-250 dye reagent: The commercially available concentrate from Bio-Rad (Hercules, CA) or Pierce Protein Assay Reagent, a ready-to-use reagent from Pierce (Rockford, IL), provides excellent results in this assay. One can also make up his or her own reagent according to the Bradford Method *(7)*. Dye reagent should always be stored at 4°C in a brown bottle.
2. Ovalbumin and γ-globulin standards: Dissolved at 5 mg/mL in "urea mix" (9 *M* urea, 4% [v/v] NP-40, 2% [w/v] ampholine, 2% mercaptoethanol) or equivalent sample buffer. Store the standards in aliquots at –20°C to ensure reproducibility. Ovalbumin and γ-globulin should be >98% electrophoretic pure. Standards are good for 6–8 mo when stored at –20°C and for up to 1 yr at –80°C.
3. Water used in making all reagents should be of very high purity, as available from Nanopure or Milli-Q 18 MΩ resistivity systems. All tubes and pipets used should be plastic, since plastic does not bind the dye reagent as well as glass. Additional reagents should be ACS grade or better.

3. Methods

The assay will take approx 2 h to complete. More experienced lab personnel will finish the assay in less time.

1. Remove ovalbumin standard aliquots (*see* **Note 1**) from the –20°C freezer and the "urea mix" from the –80°C freezer to thaw. Once thawed, spin the ovalbumin solution at 12,000*g* in the microfuge for 3 min to clarify the sample.
2. Clarify (microfuge, 12,000*g*) the unknown samples for 20 min as above before taking an aliquot for protein determination.
3. Freshly prepare a mixture of 0.1 *N* HCl (10 µL/tube) and deionized, distilled, nanopure (ddn) H_2O (80 µL/tube). Make sufficient to complete 6–10 more assays than you actually need. Cover the mixture with parafilm until ready to use.
4. Dilute 1 vol of Bio-Rad dye concentrate with 3 vol ddH_2O. Do not mix the dye vigorously. Cover the diluted dye with parafilm until ready to filter. If the Pierce

Table 1
Recipe for Ovalbumin Standards

µg Protein	5	10	15	20	25	30	40	50
µL Std. sol.	1	2	3	4	5	6	8	10
µL Urea mix	9	8	7	6	5	4	2	0
µL 0.1 *N* HCl	10	10	10	10	10	10	10	10
µL H$_2$O	80	80	80	80	80	80	80	80

   Protein Assay Reagent is used, bring the reagent to room temperature before using it as is. Figure in about 10–20 extra milliliters in order to have enough; 3.5 mL dilute dye will be added to each tube.

5. Make several dilutions of ovalbumin standard containing from 5 to 50 µg/µL "urea mix" to generated a standard curve (*see* **Table 1** as an example) (*see* **Note 1**). Always wipe the outside of pipet tips before dispensing solution to the bottom of each tube. Prepare standard and samples in triplicate (*see* **Notes 2–4**). Dilute all standards and samples to 10 µL with "urea mix." Add 90 µL of freshly diluted 0.1 *N* HCl from **step 3**. Vortex mix tubes gently.
6. Filter the working dye reagent solution through Whatman no. 1 or equivalent paper.
7. Add 3.5 mL of filtered dye reagent to each tube, and vortex mix gently.
8. After 5 min, read absorbance at 595 nm. Glass or plastic cuvets are acceptable, but disposable cuvets are preferable.
9. Graph a standard curve (**Fig. 1**) (*see* **Note 8**).

4. Notes

1. Ovalbumin or γ-globulin is used as the standard protein, because BSA absorbs twice as much dye as most other proteins. If BSA is used as the standard protein, protein concentration of unknown samples will be underestimated when compared to values estimated by Lowry. However, BSA is a satisfactory standard if one is studying serum proteins.
2. All solubilizations in buffers containing high concentrations of urea should be done at room temperature to prevent carbamylation of proteins. Carbamylation of proteins will produce altered isoelectric points ("carbamylation train") during isoelectric focusing and increase the molecular weight of protein(s) *(10)*.
3. Ovalbumin is dissolved at room temperature in "urea mix" at 5 mg/mL for at least 2 h or overnight. Ovalbumin from Sigma (St. Louis, MO), has been very reproducible.
4. Experimental samples are dissolved at a concentration of 5–10 µg lyophilized dry wt/µL urea mix. Tissue-culture samples are dissolved at 2×10^6 cells/150 µL urea mix. Other samples are dissolved at appropriate concentrations to obtain approx 5 µg protein/µL urea mix. The purpose for 5 µg protein/µL is to have between 6 and 30 µL of sample to load on the ISO gel.
5. The author has found that using a triangular magnetic spin vane in 1.5-mL skirted microfuge tubes facilitates dissolving of sample. They can be grouped standing in the middle of a stir plate.
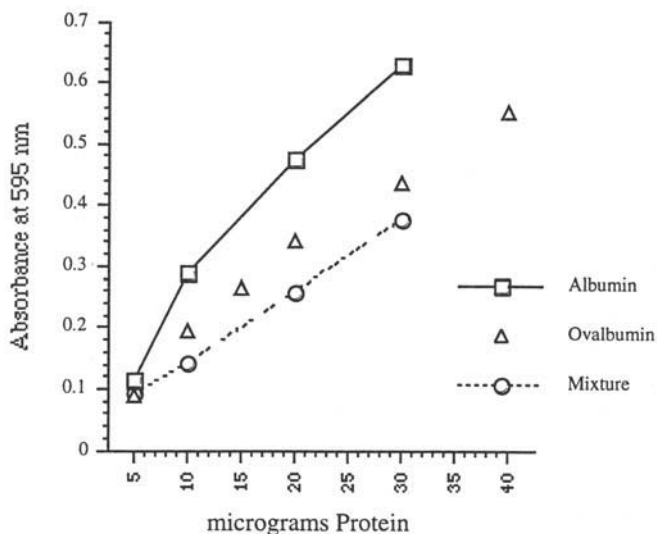
Fig. 1. Comparison of response of BSA (□), ovalbumin (Δ), and Pharmacia's low-mol-wt (○) protein standards in the modified Bradford assay. Each point represents the mean of three determinations.

6. Protein concentration in buffers containing SDS cannot be determined in this assay. However, buffers containing up to 0.3 M NaCl and 1.6 mg/mL protamine sulfate will produce linear response, but have a lower slope compared to "urea mix" *(1)*. The lower slope will make small differences in concentration more difficult to determine.
7. Use 70% ethanol to rinse dye out of glass cuvets and other glassware before detergent washing.
8. Draw a curve that fits your points instead of a straight line to get the most accurate results. It compensates for slight nonlinearity.

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13

Measuring the Radioactivity of 2-D Protein Extracts

Andrew J. Link and Nick Bizios

1. Introduction

The in vivo radioactive labeling of proteins is used to enhance the sensitivity of detection and to quantitate the abundance individual proteins. To compare the quantity and identity of resolved proteins using 2-D gels, identical amounts of sample must be loaded. It is therefore a necessity to measure accurately the radioactivity of 2-D protein extracts to determine the amount of each sample to load onto the 2-D gel. The protocol for measuring the number of counts is achieved by differentially precipitating the protein products with trichloroacetic acid (TCA) from the unincorporated radioactive precursor, washing away the precursor, and measuring the radioactivity of the precipitate using a scintillation counter. This chapter provides two protocols for quantifying the incorporation of radioisotopes during in vivo labeling. The first protocol filters the TCA-precipitated proteins onto a glass filter fiber and the second directly measures the TCA-precipitated proteins. This chapter is provided as a handy reference for the novice wishing to run 2-D gels using radiolabeled cell extracts.

2. Materials

2.1. Equipment

1. Whatman GF/C glass-fiber filters (25 mm diameter).
2. Filtration apparatus: a 25-mm glass filter holder and support with a 125-mL filter flask (Millipore, Bedford, MA, cat. no. XX10 025 00).
3. Scintillation counter.
4. Scintillation vials.

2.2. Reagents

1. 5% TCA solution: 5 g of TCA dissolved in dH_2O to a final volume of 100 mL. Store at room temperature. Since TCA is extremely caustic, protect eyes and avoid contact with skin when working with TCA solutions.
2. 20% TCA solution: 20 g of TCA dissolved in dH_2O to a final volume of 100 mL. Store at room temperature.
3. 50% TCA solution: Mix 50 g of TCA in dH_2O to a final volume of 100 mL. Store at room temperature.
4. BSA solution: 10 mg/mL BSA: 10 mg of BSA dissolved in dH_2O to a final volume of 1 mL. Store at –20°C.
5. Sample buffer solution (SB): 9.95 *M* urea, 4.0% Nonident P-40 (NP40), 2% pH 6.0–8.0 ampholytes, 100 m*M* dithiothreitol (DTT). Mix 59.7 g of urea, 44.9 g of dH_2O, 4.0 g of NP40, 5.5 g of pH 6.0–8.0 ampholytes, 1.54 g of DTT in this order in a 30–37°C water bath just long enough to dissolve the urea. Filter through a 0.2-µm filter, and aliquot 1 mL into microcentrifuge tubes. Snap-freeze in liquid nitrogen and store at –70°C.
6. Coomassie stain solution: Mix 50 mL of isopropyl alcohol, 20 mL of acetic acid, 0.1 g of Coomassie stain, and 130 mL of dH_2O until dissolved, and store at room temperature.
7. TCA carrier solution: Thoroughly mix 20 mg of BSA, 10 mg of DL-methionine, 10 mL of SB solution, and 100 mL of dH_2O. Add 0.5 mL of Coomassie solution. Store in a lightproof container at 4°C for up to 4 mo.
8. Scintillation fluid (Biofluor from NEN, Boston, MA).

3. Methods

3.1. Measuring Specific Activity Using a Glass Filter Fiber

1. Spot 1–5 µL of the radiolabeled protein extract onto a glass-fiber filter, and allow to dry completely.
2. Add an equivalent volume of the radiolabeled extract to a microfuge tube containing 0.5 mL dH_2O.
3. Add 1 µL of the BSA solution and mix.
4. Add 0.5 mL of ice-cold 20% TCA solution, and thoroughly mix. Place on ice for 30 min.
5. Collect the TCA-precipitated proteins onto a glass-fiber filter using the filtration apparatus under vacuum. Rinse the microfuge tube with 1–2 mL of the 5% TCA solution, and collect any additional precipitated proteins on the filter disk.
6. Wash the filter disk four times with 5 mL of the 5% TCA solution (*see* **Note 1**).
7. Rinse the filter disk with 5 mL of ethanol.
8. Remove the disk from the holder, and allow the filter to dry completely (*see* **Note 2**). Rinse the filter apparatus with H_2O.
9. Insert the unwashed and washed filter disks into separate scintillation vials, and add scintillation fluid.
10. Measure the amount of radioactivity on each filter using a liquid scintillation counter (*see* **Note 3**).
11. Calculate the proportion of radioisotope incorporation (*see* **Note 4**).

3.2. TCA Sample Precipitation
to Determine Directly Radioisotope Incorporation

The following protocol is adapted from the published protocol of Garrels *(1)*:

1. In a microcentrifuge tube, aliquot 300 µL of TCA-carrier solution.
2. Add 2 µL of your sample, and briefly vortex.
3. Add 150 µL of the 50% TCA solution, and briefly vortex.
4. Centrifuge at 16,000*g* for 2 min at room temperature.
5. Carefully aspirate the supernatant making sure not to disturb the pellet (*see* **Note 5**).
6. Add 1 mL liquid scintillation fluid.
7. Thoroughly mix by vortexing, and count accordingly.

4. Notes

1. During washing, the unincorporated radioactive precursors are washed from the filter, and radioactive proteins are trapped on the filter.
2. The drying time can be decreased using a heat lamp.
3. To count the incorporation of ^{3}H, ^{13}C, and ^{35}S, it is necessary to use toluene-based scintillation fluid. The efficiency of counting varies depending on the instrument and needs to be determined for the counter used. Cerenkov counting using the ^{3}H channel of a liquid scintillation counter can count ^{32}P on the dry filters. However, the efficiency of measuring Cerenkov radiation varies depending on the counter, the geometry of the scintillation vials, and the amount of water remaining on the filters. The efficiency of Cerenkov counting using dry filters is approx 25%. However, ^{32}P can be measured with 100% efficiency by adding toulene-based scintillation fluid to the dried filters and counting in the ^{32}P-channel of the scintillation counter.
4. To determine the proportion of isotope incorporation, the amount of radioactivity on the unwashed filter is compared to the amount on the washed filter: proportion incorporated = (cpm in washed filter ÷ cpm in unwashed filter).
5. The pellet can be washed with 150 µL of 10% TCA solution to remove unincorporated label if desired.

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14

Advantages and Disadvantages of Carrier Ampholyte IEF

Mary F. Lopez

1. Advantages and Disadvantages of Carrier Ampholytes

There are several advantages to the use of carrier ampholytes for running IEF separations. Tube gels using carrier ampholytes are easy to prepare and do not require sophisticated gradient casting equipment. Ampholyte mixtures can be simply blended and optimized for wide or more limited pH ranges. Blends can be engineered to be linear throughout the pH range or sigmoidal to emphasize a particular pH region *(1)*. Protein resolution is exquisite when very thin tube gels are used and the reproducibility of the gradients can be excellent *(2–4)*. Some of the disadvantages of carrier ampholyte IEF have centered on batch-to-batch reproducibility of the ampholytes themselves, since the chemical synthesis procedure is long and complicated. On the other hand, the same caveat exists for immobilized pH gradients, since each new batch of IPG acylamido/immobilines is subject to lot-to-lot variability as well. Another disadvantage of carrier ampholytes is that although theoretically the pH gradient is stable when it reaches "equilibrium," in reality, the phenomenon of cathodic drift owing to electroendosmosis in the gel causes an eventual breakdown of the basic end of the pH gradient with time *(5)*. However, the effect can be minimized if the IEF separations are standardized with respect to volt hours. If very basic proteins (i.e., with p*I*s above 8.0) are to be separated, nonequilibrium pH electrophoresis or NEpHGE, should be employed.

In the past, many researchers created their own 2-D systems, and this heterogeneity led to a lack of standardization in the resulting 2-D gel patterns. Much of this irreproducibility can now be avoided by use of some of the commercially available dedicated 2-D systems *(1)*. Variables such as temperature,

prefocusing and focusing algorithms, and gel chemistry have been standard-ized for consistent results from run to run *(6,7)*. Carrier ampholytes have been optimized to yield reproducible results (to 0.016 p*I* units) from lot to lot and gel to gel *(3)*. In addition, improvements, such as threaded tubes for IEF *(1)* and high-purity, 2-D electrophoresis chemicals, allow the once difficult tech-nique of 2-D electrophoresis to become routine in any laboratory.

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15

2-D Electrophoresis Using Carrier Ampholytes in the First Dimension (IEF)

Mary F. Lopez

1. Introduction

Two-dimensional electrophoresis (2-DE) is potentially the most powerful technique known for resolving complex protein mixtures. Proteins can be purified to homogeneity in one step in most cases. The applications for 2-DE are numerous and diverse. They include the analysis of protein patterns in various organisms and tissues, monitoring of the purity of fractions derived from chromatography (or other separation methods), and the determination of the characteristics of a particular protein, such as isoelectric point, molecular weight of subunits, and number of isoforms.

The combination of 2-DE with automatic amino acid sequencers and mass spectrometry has allowed direct access to sequence information from single polypeptide spots on gels *(1,2)*. Amino acid sequences from proteins of interest can provide information on the potential function of the protein through protein database homology searches as well as clues to protein secondary structure, conformation of potential catalytic sites, and posttranslational modifications *(3,4)*. Partial amino acid sequences can also be used to synthesize nucleic acid probes for gene cloning.

High-resolution 2-DE separates proteins by charge (isoelectric focusing or IEF) in the first dimension and by relative mobility (SDS-PAGE) in the second dimension. Conventional one-dimensional (1-D) SDS-PAGE and 1-D IEF are each capable of resolving only approx 100 of the most abundant proteins in a heterogeneous sample. Employing both techniques in a 2-D separation theoretically results in the resolution of 10,000 individual spots (the product of the two techniques), since protein charge and molecular weight are independent

properties *(5)*. In practice, high-resolution 2-DE systems can resolve on the order of 3000 proteins, depending on the sample, although up to 10,000 spots have been reported on some gels *(6)*.

Traditionally, 2-DE has included the IEF first-dimension separation realized in tube gels. The IEF charge separation is achieved by adding a mixture of carrier ampholytes to the first-dimension gel solution. Typically, the carrier ampholytes are a heterogenous mixture of aliphatic polyaminopolycarboxylic acids with pH values that can range from 2.5 to 11 *(7)*. The ampholyte mixture sets up the pH gradient in the IEF gel as current is applied. An alternative to IEF with carrier ampholytes is the more recently developed immobilized pH gradient chemistry *(8)*, in which the ampholytes are covalently linked to the acrylamide and the gradient is fixed in the gel. The advent of immobilized pH gradients (IPG) has prompted debate regarding the relative advantages of traditional IEF in carrier ampholytes vs IEF in IPG *(8,9)* (*see* Chapters 14 and 19).

2. Materials
2.1. Electrophoresis Equipment

In order to run large-format 2-D gels, you will need to assemble the following equipment. It may be convenient to purchase all the components together as a dedicated system (*see* **Note 1**).

1. Power supply: The power supply must be able to deliver high voltages (2000 V) and very low current (µA) accurately for IEF separations. It is also desirable to utilize a power supply that can integrate voltage and time to deliver a consistent number of volt-hours for the IEF run. For second-dimension slab gel separations, higher current (50–100 mA) and lower voltage (500 V) will be needed.
2. 1-D casting system: IEF tubes can be cast most reproducibly by using the water-displacement method *(5,10)*. This method casts several gels at a time and fills the tubes from the bottom for even gel height. The casting system requires a vessel to hold approx 1 L of water, and a funnel with a detachable vial on the end to hold the acrylamide solution and tubes (*see* **Fig. 3**). The funnel should have a small hole drilled in the spout to allow water to enter the vial.

    A simplified casting system for casting IEF tubes individually can consist of glass capillary tubes (1–3 mm id), parafilm for sealing the bottom, and a syringe with a long, fine needle to introduce the gel solution. The thinner the tubes, the finer the resolution.
3. 1-D running system: The 1-D running tank should accommodate several tube gels in a vertical configuration with an electrolyte reservoir at the top and one at the bottom of the device, for anode and cathode solutions.
4. 2-D casting system: Casting the second-dimension slab gels can be done individually or in a box that casts several at a time. Vertical slab-gel-casting boxes are available from several manufacturers (*see* **Note 2**). If single gels are to be cast, the minimum requirement would be glass plates, spacers, and clamps for holding the assembly together.
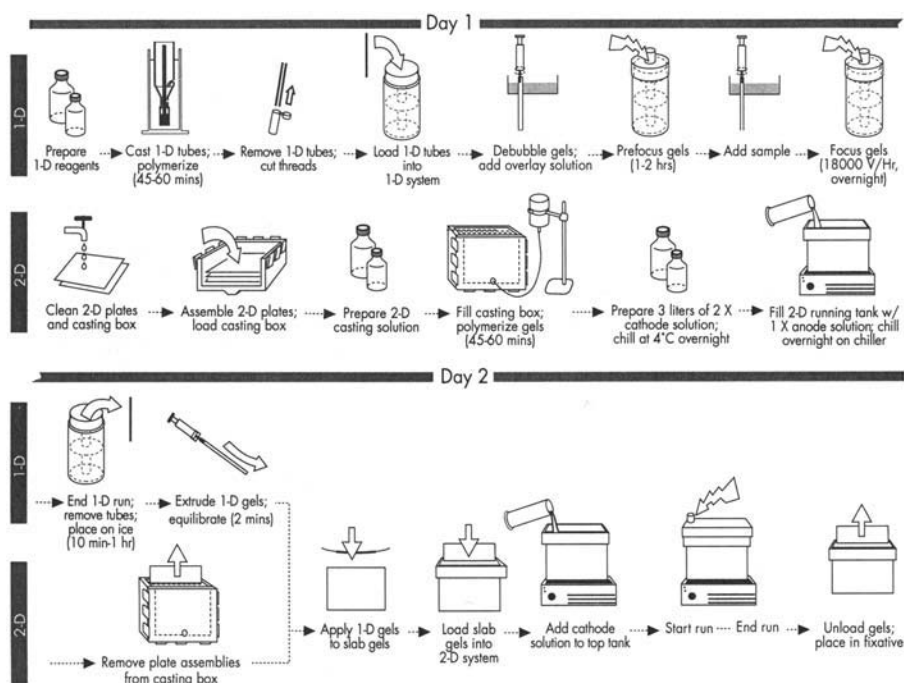
Fig. 1. Overview of 2-DE. 2-DE using carrier ampholytes in the first dimension (IEF) (Mary F. Lopez).

5. 2-D running system: The 2-D running tank should accommodate the vertical slab gel or gels and buffer reservoirs. Typically when running 2-D gels, at least two gels (and usually more) are run simultaneously to increase reproducibility.
6. Cooling system: In order to minimize diffusion and therefore spot size, the second-dimension gels should be cooled while running (*see* **Note 3**).

Overview: The steps in Fig. 1 show a typical operating sequence for a 2-D run. The Investigator System components are used in this example. However, the steps would be very similar for any 2-D system. Detailed instructions for these procedures will follow. The prefocusing step is optional.

2.2. Reagents

Use only 18 MΩ water for preparing all solutions, and filter through 0.45-μ*M* filters. Electrophoresis chemicals are available from a variety of suppliers, for example, Bio-Rad (Hercules, CA), Pharmacia (Piscataway, NJ), ESA, Fluka (Chelmsford, MA), and Sigma (St. Louis, MO). All chemicals used in the procedures should be ultrapure or "2-DE-grade," since IEF is very sensitive to impurities *(11)*. Carrier ampholytes (*see* **Note 4**) are typically sold as a 40% solution in various narrow and wide-range mixtures.

1. Acrylamide stock solutions:
   a. 30% Acrylamide, 0.8% *bis* (premixed acrylamide/*bis* solutions are available from several manufacturers, such as ESA, Bio-Rad, National Diagnostics [Atlanta, GA], Amresco [Solon, OH]).
   b. High tensile strength acrylamide/*bis* solution, such as 30% Duracryl™, 0.65% *bis* (*see* **Note 5**).
2. 1-D IEF gel solution for analytical gels: 4.1% of a stock solution of 30% acrylamide, 0.8% *bis*, 9.5 M urea, 2.0% Triton X-100, and 5 mM CHAPS. Warm the solution to 37°C to dissolve the urea, but do not heat above 37°C to prevent protein carbamylation and resulting charge artifacts. Filter the solution, and aliquot into small volumes. The solution may be stored frozen. When thawing, do not heat above 37°C.
3. IEF cathode (upper) solution: 100 mM NaOH in H_2O. The solution should be degassed prior to use (*see* **Note 6**).
4. IEF anode (lower) solution: 10 mM phosphoric acid in H_2O.
5. 1-D IEF gel equilibration buffer: 0.375 M Tris-HCl, pH 8.8, 3.0% SDS, 50 mM DTT, and 0.01% bromophenol blue. Filter and store frozen. Thaw small aliquots as needed.
6. Slab-gel buffer: 1.5 M Tris-HCl, pH 8.8, filtered. Store at 4°C.
7. 2-D gel running buffer: A 10X solution consists of 250 mM Tris, 1.92 M glycine, and 1% SDS. Filter the 10X stock solution, and store at room temperature. Dilute to make 1 or 2X buffer as needed.
8. Sample buffer mix: 40 mM Tris-HCl, pH 8.0, 8.0 M urea, 0.06% SDS, 1.8% ampholytes, 120 mM DTT, and 3.2% Triton X-100. This is a buffer for loading all types of samples for IEF. The samples should preferably be either dried down and resuspended, precipitated and resuspended, or dialyzed into this buffer before loading onto the IEF gels (*see* **Note 7**). Samples **must** be salt-free for successful IEF.
9. Water-saturated isobutanol overlay for casting slab gels: Mix equal volumes of 18 MΩ water and isobutanol in a glass bottle, and allow the phases to separate. An excess of water beneath the isobutanol phase should be retained to keep the organic layer saturated with water.
10. 10% Ammonium persulfate (APS) in H_2O.
11. Carrier ampholytes, 40% stock solution (*see* Note 4).
12. TEMED.

3. Methods

3.1. Casting IEF Gels

The following protocol is based on the water-displacement casting procedure and uses IEF capillary tubes that have a thread running through the center to allow easy handling of the IEF gel (*see* **Note 8**). *See* **Note 9** for instructions on how to cast individual tube gels.

Wear gloves for all procedures handling IEF tubes or equipment to avoid protein contamination. The IEF threaded tubes for analytical gels are packaged

in 3 bundles of 6/pack. The bundles consist of three threaded tube pairs held together with a shrink-wrap band (tube dimensions are 26 cm L × 1 mm id). You may cast 6, 12, or 18 tubes at one time. The resulting gels are 18 cm in length. Rinse the tubes in 18 MΩ H_2O before use, and gently tap to remove excess water before casting gels.

1. Prepare and thaw a 5.65-mL aliquot of the 1-D IEF gel solution. Place in a snap-cap vial that will fit securely on the end of the casting funnel.
2. Pour 1100 mL of 18 MΩ H_2O into the 1-D casting cylinder. The water temperature should be between 20 and 25°C to avoid precipitation of urea.
3. Add 350 μL of 40% ampholyte stock solution and 40 μL of 10% APS solution to the aliquot of 1-D gel solution in the vial.
4. Mix the solution completely by inverting the vial several times. Try to minimize detergent bubbles when mixing.
5. Place one to three bundles of IEF tubes with the shrink-wrapped end facing down into the vial (*see* **Fig. 2**). Tilt the vial gently to one side as the tubes are placed into the solution to avoid the formation of air bubbles.
6. Guide the small end of the 1-D casting funnel over the tops of the tubes, and press the snap-cap vial onto the funnel securely.
7. Slowly lower the snap-cap vial, tubes, and funnel combination into the casting cylinder so that the water entering through the lower hole in the funnel lays over the gel solution in the vial (*see* **Fig. 3**). Lower slowly to avoid mixing the solution.
8. Allow the funnel and vial combination to settle to the bottom of the cylinder.
9. Wait about 1 h for the gels to polymerize.
10. Slowly lift the funnel and vial combination from the casting cylinder, and allow the water to drain while lifting.
11. Separate the snap-cap vial and tubes from the funnel.
12. Carefully remove the tube bundle(s) and the attached polyacrylamide plug from the snap-cap vial.
13. Separate the tube bundles (each now containing gel) by pulling sideways as shown in **Fig. 4**.
14. Use a single-edge razor to cut the shrink-wrap tubing carefully from the bundle. The three pairs of tubes in each bundle can now be separated. Cut the thread at the top of each pair to separate the single tubes, and then cut the thread at the base to approx 2 cm from the base.

3.2. Running IEF Gels

The components of a typical 1-D running system are illustrated in **Fig. 5**. The following procedure uses a cylindrical 1-D running tank (such as The Investigator) as an example. Several different styles of running tanks are available from electrophoresis equipment manufacturers, such as Bio-Rad and Pharmacia.

1. Prepare anode and cathode solutions as described in **Subheading 2.2.**
2. Pour 2 L of anode solution into the lower reservoir of the 1-D running tank, and lower the 1-D running rack into the anode solution.
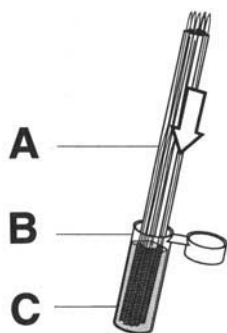
Fig. 2. Adding the capillary tubes to the vial containing 1-D casting solution.
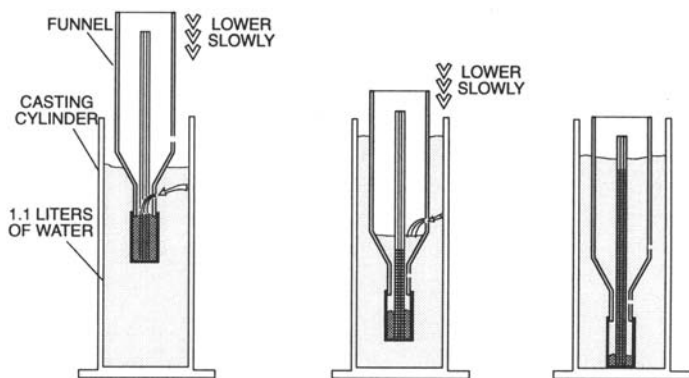
Fig. 3. Displacing the 1-D gel casting solution.

3. Slide a grommet onto each IEF tube so that the grommet is at the top of the tube. Slide the tube through a guide hole in the rack, and press down on the grommet to seal.

4. Install as many IEF tubes into the 1-D running system as you wish to run. All other empty analytical and preparative holes should be plugged.

5. Fill the upper reservoir with 800 mL of degassed cathode solution.

6. Debubble the tubes by filling a 1-mL syringe with a long, fine needle attached to cathode solution. Lower the needle to the gel surface of a tube, and expel the cathode solution until all the bubbles are released.

   At this point in the procedure, traditional IEF methods require a prefocus step. However, eliminating the prefocusing step results in improved pattern resolution (unpublished data). Therefore, in this protocol, the prefocusing step has been omitted (*see* **Note 10** for optional prefocusing protocols).

7. Using a Hamilton syringe with a fine needle, load 1–50 µL of sample in each tube. Optimum results for silver-stained gels are obtained when loading approx
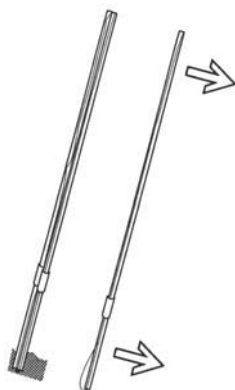
Fig. 4. Separating the tube gel bundles.

30–100 µg protein. Samples should be salt-free and prepared in sample buffer mix (*see* **Subheading 2.2.** and **Note 7**).

8. Install the cover on the 1-D running system.
9. Program the power supply with the following parameters:
    a. Maximum voltage: 1000.
    b. Duration: 17 h, 30 min.
    c. Maximum current (mA) per gel: 110.
    d. Total volt-hours: 17,500.
10. Focus the gels. If desired, the gels may be prefocused to 1000 V before loading sample (*see* **Note 10**). Also, a short period (30 min) of high voltage (2000 V) applied at the end of the run will tighten the protein zones to give even better resolution.
11. After the focusing, proceed with the following steps to extrude the 1-D tube gels. However, before you start the gel-extrusion procedure, cast the second-dimension slab gels, allow them to polymerize, and remove them from the casting box if casting more than one gel at a time (*see* **Subheading 3.3.**).
12. Remove the tubes from the 1-D running system. The cathode solution will drain into the anode solution.
13. Place the tubes on ice (to stiffen the gel) for approx 5–10 min. The tubes can remain on ice for up to 20 min.
14. Prepare and thaw the 1-D gel equilibration buffer as described in the **Subheading 2.2.** Gels can be extruded and frozen for later use (*see* **Note 11**).
15. Fill a 1-mL IEF gel-extrusion syringe and extrusion adapter (*see* **Note 12**) with 18 MΩ H_2O, and attach an IEF tube (with the thread flush with the top of the tube). Turn the adapter nut until it is tight.
16. Fill a holding tray with 1-D gel equilibration buffer.
17. Exert constant pressure on the syringe plunger until the end of the gel begins to move out of the tube (*see* **Fig. 6**).
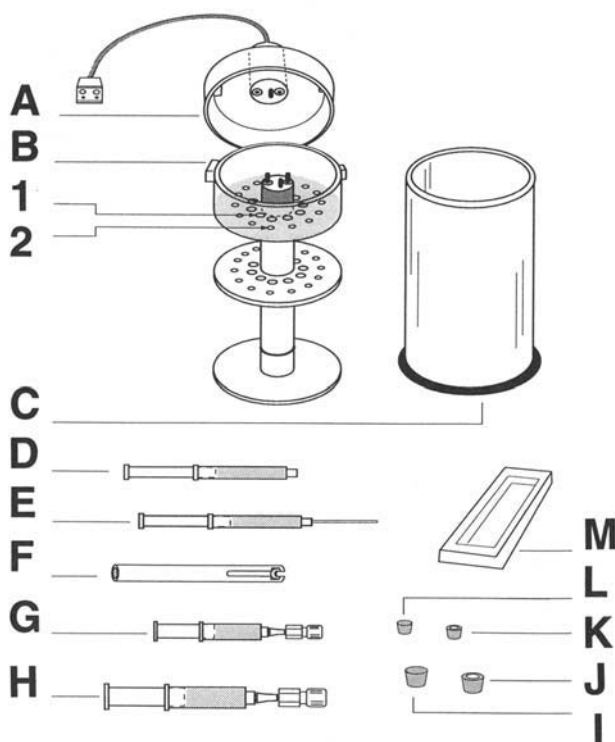
Fig. 5. Parts of the Investigator 1-D running system.

18. Decrease the pressure, and slowly extrude the gel into the holding tray with buffer.
19. Incubate the gel in the equilibration buffer for 2 min at room temperature, and then proceed to load it onto a slab gel (*see* **Subheading 3.4.**).
20. Repeat **steps 6–12** until all the gels have been extruded, incubated in the buffer, and then loaded onto the slab gels.

3.3. Casting 2-D Gels

The following instructions describe how to cast from one to six gels at a time in a casting box using the Investigator slab-gel-casting box as an example. *See* **Note 2** for instructions for casting individual gels.

A maximum of six, 20 × 20 cm, 1.0-mm thick 2-D slab gels can be cast at one time in the standard casting box. If fewer than six gels are to be cast, spacer blocks must be used to fill the remaining space. The slab-gel casting assembly is filled from the bottom so that all of the gel cassettes will be filled uniformly (*see* **Fig. 7**).

1. Place the casting box on its back so that the front opening is facing up and the bottom of the box is at the front of the bench.
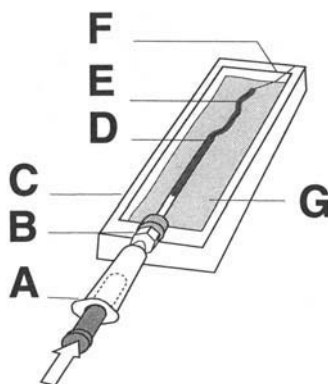
Fig. 6. Extruding the tube gel.
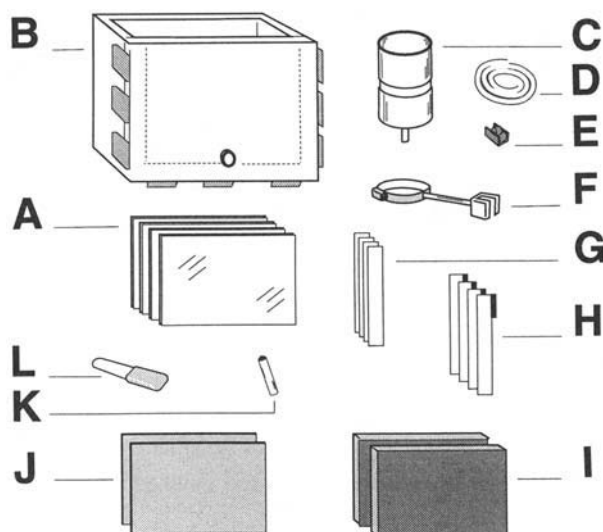
Fig. 7. Parts of the 2-D slab gel casting system.

2. Fold a sheet of polyethylene film (*see* **Note 13**) in half, and place it with the fold along one edge in the box. The top edge of the folded sheet must extend at least 0.5 cm above the top of the casting box and gel plates (*see* **Fig. 8**).
3. Wearing gloves, place a glass plate on the bench cover with the bevel (*see* **Note 14**) at the top and facing up. Wipe the plate with 95% ethanol, and dry with lint-free paper towels.
4. Use a syringe filled with petroleum jelly (*see* **Note 15**), and apply a bead to one surface of each spacer along the outside edge. Position the spacers so that the side with the jelly contacts the glass plate.
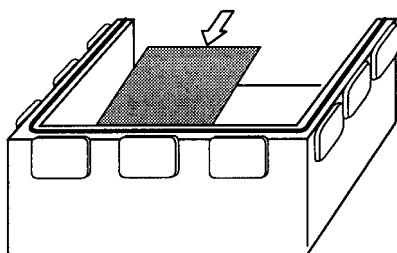
Fig. 8. Assembling the 2-D casting box.
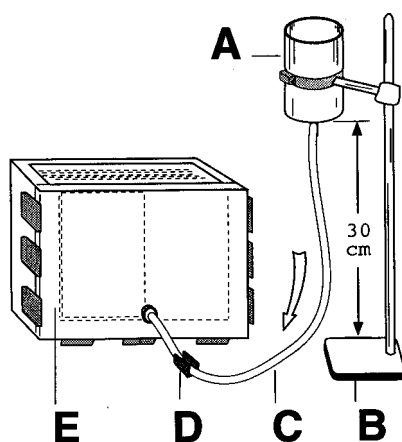
Fig. 9. Setting up the 2-D casting box.

5. Apply a bead of jelly to the opposite surface of the spacers, and place a second glass plate on the spacers with the top bevel facing down.
6. Place the completed glass cassette in the casting box with the beveled edge at the top of the box.
7. Place another folded sheet of polyethylene film on top of the cassette just installed. The second sheet should be on the alternate side from the first. All sheets should extend approx 0.5 cm above the top of the plates and casting box.
8. Repeat **steps 4–10** until the casting box has the desired number of cassettes in place. Alternate sides with the folded polyethylene sheets. Do not put a polyethylene sheet on top of the last cassette.
9. After the cassettes are installed, place an appropriate number of foam spacer pads (*see* **Note 16**) to bring the level of the cassettes up to the edge of the box. Clamp the front cover onto the casting box, stand the casting box upright, and level the box.
10. Attach the casting cylinder (*see* **Note 17**) to a sturdy stand or rack so that the bottom of the cylinder is at least 30 cm above the benchtop (*see* **Fig. 9**).
11. Connect the casting cylinder to the casting box with tubing.
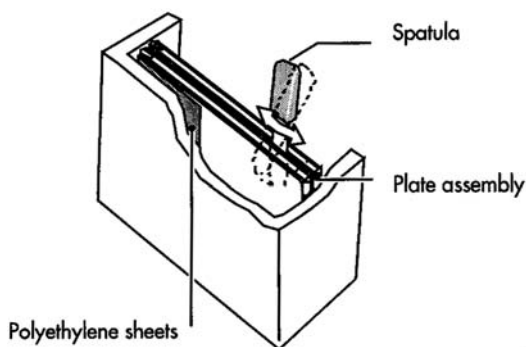
Fig. 10. Disassembling the 2-D casting box.

12. Prepare the acrylamide solution using the following procedure (refer to the chart below for the specific volumes of each solution):
    a. Mix the following solutions:
       Duracryl (*see* **Note 5**), 30.65% T.
       1.5 *M* Tris slab-gel buffer.
       18 MΩ H₂O.
    b. Degas the mixture for 10 min.
    c. Add the appropriate volume of 10% SDS stock solution.
    d. Initiate polymerization by adding TEMED and 10% ammonium persulfate.
    e. Swirl gently to mix, and pour into the casting cylinder.
13. Allow the casting box to fill with acrylamide solution until the level has reached to within 0.5 cm from the bottom of the bevel between the paired glass plate cassettes.
14. Close the clamp, and carefully layer 0.5 mL of water-saturated butanol onto the top of the acrylamide solution of each cassette.
15. Allow the solution in the casting box to polymerize for 60 min.
16. Once polymerization is complete, separate the cassettes by carefully inserting a wide-blade spatula between the folded polyethylene sheets separating the adjoining cassettes. Use force to push the spatula straight down in between the folded sheet separating each cassette (*see* **Fig. 10**). Remove excess polyacrylamide from the outside of the cassette by rinsing the cassettes with water. Use a laboratory squirt bottle filled with 18 MΩ water to rinse the surface of the gel between the beveled plates.

3.4. Running the 2-D Gels

The following procedures describe how to run the second-dimension slab gels using the Investigator System as an example. Vertical slab-gel apparatuses from other manufacturers operate in a similar fashion. Bear in mind that cooling the gels while they run is necessary to ensure high resolution (*see* **Note 3**). In the following example, a Peltier chiller (*see* **Fig. 11**) serves this function. Other cooling apparatuses, such as recirculating chillers or running the gels in
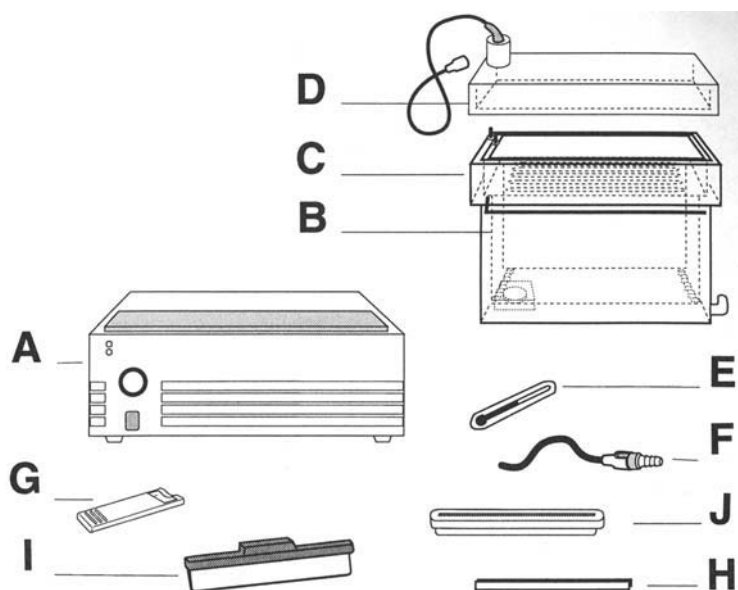
Fig. 11. Parts of the 2-D running system.

a cold room, can also be used. The 2-D running system described below runs a maximum of five single or five double (total 10 gels) slab gels.

1. Place the 2-D running tank on the chiller plate with the stir bar and the electrode posts on the left side of the chiller.
2. Fill the lower chamber of the tank with 10.0 L of 1X 2-D gel running buffer (*see* **Subheading 2.2.**).
3. Turn the chiller on, and set the dial to maximum. Cool the lower buffer for at least 1 h or until it reaches a temperature of 10°C.
4. Prepare 3 L of 2X Tris running buffer and prechill to 4–10°C. Placing the buffer overnight in a refrigerator or cold room is convenient.
5. Fill any unused slots in the running tank with gasket plugs.
6. Place the slab gels in a rack on the benchtop (*see* **Note 19**), and fill the top of each gel with 2X 2-D gel running buffer from a laboratory squeeze bottle.
7. Carefully lift an equilibrated tube gel by the thread ends (**Note 8**).
8. Place the tube gel on top of a second-dimension slab gel (*see* **Fig. 12**). It should fit easily in the V-shaped trough formed by the bevels of the glass plates. Trim off the long ends of the thread with scissors.
9. Gently slide the tube gel down onto the surface of the slab gel using the gel installer (*see* **Fig. 13**, **Note 20**). A stream of running buffer from a laboratory squeeze bottle is useful in positioning the tube gel onto the slab gel. Make sure there are no air bubbles trapped in between the tube and slab gels.
10. Repeat **steps 1–4** until all the gels are loaded.
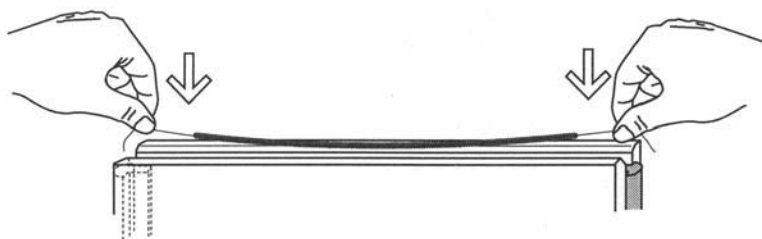
Fig. 12. Positioning the IEF tube gel.
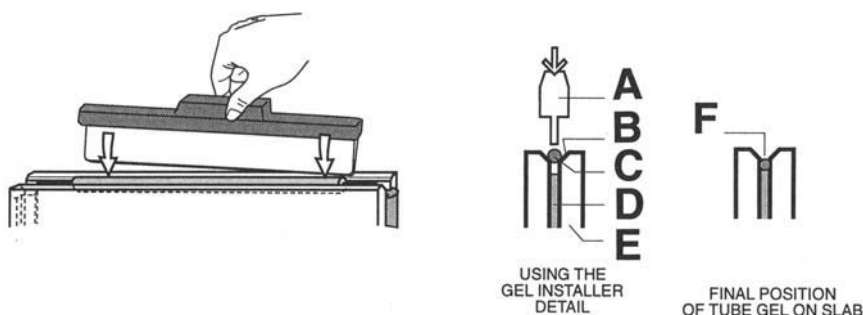
Fig. 13. Using the gel installer to place the 1-D tube gel on the slab gel.

11. Wet a sealing gasket (**Note 21**) with running buffer. Slide the gasket over the top of a slab-gel cassette.
12. Install the cassette through a slot in the 2-D tank rack.
13. Seat the sealing gasket in the slot by pressing the ends of the gasket first and then pressing toward the middle.
14. Repeat **steps 11–13** for each gel cassette.
15. Fill the upper buffer chamber with 3 L of 2X gel running buffer (*see* **Note 22**).
16. Place the cover on the 2-D running system, and program the power supply with the following parameters:
    a. Max voltage (V): 500.
    b. Max current (mA) per gel: 75.
    c. Max Power (W) per gel: 20 (*see* **Note 23**).
17. The 2-D run is stopped manually when the bromophenol blue dye front reaches within 1 cm from the bottom of the gel. When the run is completed, drain approx 3 L of buffer from the bottom tank.
18. Remove the gel cassettes. Allow the buffer in the upper tank to drain into the lower tank. The mixture of anode and cathode buffers resulting in the lower tank may be reused six to seven times.
19. Using a spatula, open the gel cassettes, and place the gels in fixative solution or transfer buffer if they are to be electroblotted to membranes.

4. Notes

1. To simplify the 2-D process, it is convenient to use a "dedicated 2-D System," such as The Investigator™ (ESA) or Iso Dalt™ (Hoefer-Pharmacia). The Investigator includes the components described below, and is used in many of the examples and procedures given in this chapter.

   a. Power supply: The programmable, multitasking power supply has built-in algorithms or IEF prefocusing and focusing to supply a consistent number of volt hours during the separations.

   b. 1-D casting system. From 6 to 18 analytical IEF tube gels (and 1–8 preparative gels) can be cast using an acrylamide/ampholyte solution and water displacement. Analytical gels are cast in 26-cm tubes to a height of 18 cm and a diameter of 1.0 mm. Each analytical tube has a thread running through the center, which allows easy handling of the tube gels once they are cast and extruded.

   c. 1-D running system: The cylindrical 1-D running system performs isoelectric prefocusing and focusing separations on 1–15 analytical tube gels or 1–8 preparative tube gels.

   d. 2-D casting system: The 2-D casting system is used to cast from one to six second-dimension slab gels using a bottom-fill gravity feed system. The resulting gels are 20×20 cm $\times 1$ mm thick.

   e. 2-D running system: The 2-D running tank performs SDS-PAGE separation on up to five slab gels. A ceramic bottom on the tank allows efficient cooling when placed on top of the chiller.

   f. Chiller: The Peltier chiller maintains buffer temperatures at 10–20°C in the second-dimension tank (depending on power load and environmental conditions).

2. Vertical slab-gel-casting equipment is available from Bio-Rad, Pharmacia, Owl Scientific, ESA, and most electrophoresis equipment suppliers. To cast individual gels, place thin (1–3 mm) spacers between glass plates on the sides and bottom, and secure with binder clips. A small amount of vaseline or water-soluble glue can be used on the spacers to prevent leaking of acrylamide solution. Place the glass cassette upright on a bench and fill with acrylamide solution to within 0.5–1 cm of the top. Immediately overlay with a small volume (0.3–0.5 mL) of water-saturated butanol. Allow the gel to polymerize, and then remove the bottom spacer and rinse the cassette with 18 MΩ water.

3. Cooling the second-dimension gels can be simple or sophisticated. If one is available, the second-dimension tank can be run in a cold room. However, this does not regulate the buffer temperature precisely. Several commercially available slab-gel tanks can be connected to a recirculating chiller to regulate the buffer temperature. The Investigator System utilizes a Peltier chiller to cool the second-dimension tank. The optimum temperature for running the second-dimension gels is the coolest possible without precipitation of the SDS. Practically, this is between 10 and 20°C.

4. There are several worldwide suppliers of very good-quality carrier–ampholyte mixtures. Some of the larger manufacturers are Serva, BDH, ESA, Pharmacia,

and Bio-Rad. Use wide-range ampholyte mixtures, such as pH 3.0–10.0, for complex protein mixtures. Narrow-range mixtures can help to resolve close-charge isoforms.

5. When running large format slab gels, it is convenient to use a high-tensile-strength acrylamide solution, such as Duracryl (ESA), since large format gels made with standard acrylamide can be very fragile and hard to handle for staining. The Duracryl mixture has enhanced staining and tensile-strength properties, and is compatible with preparative techniques, such as protein sequencing and mass spectrometry *(12)*.

6. The NaOH cathode electrolyte is degassed to prevent acidification owing to CO_2 dissolving in the solution and forming carbonic acid.

7. Proper preparation of samples for IEF is the key to good 2-D results. Samples Must be free of salts and other contaminants, such as pigments, phenolic compounds, and nucleic acids. Either precipitation with protamine sulfate (1 mg/mL) or digestion with DNase/RNase can eliminate nucleic acids. The samples can then be cleaned up further either by precipitation with acetone (to 80% volume) or by dialysis into a diluted sample buffer. Protein concentration must be above 1 mg/mL for good recovery when using precipitation methods. Very good results for more dilute samples are obtained by dialyzing into a 1:10 dilution of sample buffer mix and then concentrating the dialysate in a speed vac.

8. The threaded tubes allow very thin, fragile IEF gels to be handled easily, because the thread is cast into the gel and runs through it. These 1-mm-thick gels are optimal for fine resolution of polypeptide spots. No adverse effects on protein mobility or spot shape are the result of the thread *(5)*. The tubes are available from ESA Inc.

9. To cast individual IEF gels, cover the bottom of capillary glass tubes (1–3 mm id) with parafilm securely, and place tubes upright in a rack. Working quickly, use a syringe with a fine needle to fill the tubes with 1-D IEF gel solution to which catalyst has been added. Allow some space at the top of the tube for sample loading. After polymerization has occurred, rinse the tops of the gels with 18 MΩ water.

10. If you wish to prefocus the IEF gels, set a current limit of 1000 V and a time duration of approx 2 h. Prefocus the gels before loading sample. The prefocusing has completed when the voltage limit is reached, regardless of the time elapsed.

11. Best results are obtained using fresh gels. However, if necessary, you can extrude and store 1-D gels by substituting 10% glycerol in place of equilibration buffer. Gels may be extruded and stored in small Petri dishes indefinitely at –70°C. On thawing, place the gels in equilibration buffer for 2 min before loading onto slab gels.

12. The IEF gels are best extruded from the tubes with water pressure. A 1-cc syringe can be fitted with a small piece of tubing with a diameter that will fit the tube snugly. Alternatively, compression adapters for extrusion are available from electrophoresis equipment manufacturers. In the example given in the text the IEF gel extruder from ESA was used.

13. Polyethylene film is used to separate the glass cassettes in the casting box. This makes removal of the cassettes easier once the acrylamide has polymerized.

14. The glass plates used for casting gels in this procedure have a bevel ground along the top edge. When the gels are cast, the two glass plates forming the gel cassette are arranged so that the bevels face each other at the top of the cassette. This forms a small trough that makes positioning the tube gel easier.

15. Sealing the cassettes with petroleum jelly prevents current leaks along the edges of the gel, which would result in a pattern that "bows out" at the sides. Other substances, such as water-soluble glue stick, silicone grease, or Celloseal (Bio-Rad), can also be used to seal the cassettes. The more water-soluble the substance is, however, the less likely it will be to form a good electrical seal over the course of the run. Unfortunately, the non–water-soluble sealants are more difficult to clean from the glass plates. To clean the glass plates, use very hot water and a strong detergent, such as Micro Detergent (Millipore Corp. [Bedford, MA], ESA [Chelmsford, MA]).

16. When casting fewer than the maximum gels in a casting box, the extra space may be filled to prevent wasting a large amount of acrylamide solution. Almost any material may be used to make spacer blocks, such as dense foam, glass, or plastic.

17. It is convenient to have a container for holding the acrylamide solution with a port on the bottom that can be clamped shut and then opened while filling the casting box. The casting cylinder used in the example given in the text is part of the Investigator System.

18. Use different acrylamide concentrations for different mol-wt proteins. High-mol-wt proteins are best resolved with 7.5% acrylamide, and low-mol-wt proteins with 15%. Use 10–12.5% for most whole-cell protein mixtures.

19. Polypropylene test tube racks with multiple pegs (VWR [Boston, MA], ESA) work well for holding the glass cassettes in position.

20. The tube gels can be installed onto the slab gels by gently pressing down with a flat spatula. A gel installer tool can also be constructed out of a thin, flat plastic spacer that fits easily in the space between the glass plates. The best technique for loading the tube gels flat onto the surface of the slabs is by tapping straight up and down using the full width of the installer tool. Do not drag the corner of the tool along the tube gel, since this can tear the tube gel.

21. The running tank used in the example is from the Investigator. The slab-gel cassettes are placed into slots in a holding rack. The slots are sealed with gaskets to prevent current from flowing around the gels instead of through them.

22. Double strength (2X) buffer is used in the upper chamber to prevent ion depletion (Tris and SDS), which would cause streaking, especially of lower-mol-wt acidic proteins. The lower buffer is kept at 1X concentration to economize on buffer use since it is principally used to cool the gels.

23. Second-dimension gels can be run using constant current or constant power. In either case, a voltage limit of 500 V should be set to minimize overheating of the gels. If gels are run at constant current, the limit should be set to 60–70 mA/gel. Running the gels on constant power instead of constant current maintains the speed of migration consistent, since the resistance in the gel will change over the course of the run ($P = IV$, where P is power measured in watts, I is current measured in amps, and V is voltage).

References

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16

Nonequilibrium pH Gel Electrophoresis (NEPHGE)

Mary F. Lopez

1. Introduction

Nonequilibrium pH gel electrophoresis (NEpHGE) is a technique developed to resolve proteins with extremely basic isoelectric points (pH 7.5–11.0) *(1,2)*. These proteins are difficult to resolve using standard IEF, because the presence of urea in IEF gels has a buffering effect and prevents the pH gradient from reaching the very basic values (with a pH above 7.3–7.6) *(3)*. In addition, cathodic drift causes many very basic proteins to run off the end of the gel. During NEPHGE, proteins are not focused to their isoelectric point, but instead move at different rates across the gel owing to charge. For this reason, the accumulated volt hours actually determine the pattern spread across the gel. It is therefore imperative that volt hours be consistent to assure reproducible patterns.

2. Materials
2.1. Electrophoresis Equipment and Reagents

The procedures and equipment required for running NEPHGE are very similar to those for IEF. Please refer to Chapter 15 for the necessary electrophoresis equipment and reagents. In order to run the NEPHGE procedure, it will be necessary to reverse the polarity in the 1-D gel running tank. This is usually achieved with an adapter that attaches to the tank or power supply.

3. 2-D NEPHGE Protocol
3.1. Casting IEF and 2-D Slab Gels

Follow the instructions given in the Chapter 15 to cast the tube gels and the second-dimension slab gels. Use a wide-range ampholyte mixture, such as 3–10, for best results (*see* **Note 1**).

3.2. Running the NEpHGE Gels

1. Prepare anode and cathode solutions as described in **Subheading 2.2.** of Chapter 15.
2. Pour 2 L of degassed cathode solution into the lower reservoir of the 1-D running tank and lower the 1-D running rack into the cathode solution (*see* **Note 2**).
3. Slide a grommet onto each IEF tube so that the grommet is at the top of the tube. Slide the tube through a guide hole in the rack and press down on the grommet to seal.
4. Install as many IEF tubes into the 1-D running system as you wish to run. All other empty analytical and preparative holes should be plugged.
5. Fill the upper reservoir with 800 mL of anode solution (*see* **Note 2**).
6. Debubble the tubes by filling a 1-mL syringe with a long, fine needle attached with anode solution. Lower the needle to the gel surface of a tube, and expel the anode solution until all the bubbles are released.
7. Using a Hamilton syringe with a fine needle, load 1–50 μL sample in each tube. Optimum results for silver-stained gels are obtained when loading approx 60–100 μg protein. Samples should be salt-free and prepared in sample buffer mix (*see* **Subheading 2.2.** of Chapter 15).
8. Install the cover on the 1-D running system.
9. Program the power supply with the following parameters to prerun the gels:
    a. Maximum voltage: 1500.
    b. Duration: 2 h.
    c. Maximum current (mA) per gel: 110.
10. Prerun the gels until the voltage reaches 1500 V. This should occur within 2 h (*see* **Note 3**).
11. Program the power supply with the following parameters to run the gels:
    a. Maximum voltage: 1000.
    b. Duration: 4 h.
    c. Maximum current (mA) per gel: 110.
    d. Total volt-hours: 4000.
12. Cast and prepare the second dimension gels as described in Chapter 15.
13. After running the NEPHGE IEF gels, proceed to extrude them and load onto the second dimension gels as described in Chapter 15.
14. Run the second-dimension gels as described in Chapter 15.

4. Notes

1. Ampholyte mixtures are usually provided as a 40% aqueous solution. Although the NEPHGE procedure separates proteins with basic p*I*s, it is best to use a wide-range ampholyte mixture. A basic mixture of ampholytes will crowd the acidic proteins in a narrow region and potentially obscure some of the basic proteins.
2. For the NEPHGE procedure, the positions of the anode and cathode buffers are reversed. This results in a better separation of the basic proteins.
3. During NEPHGE, the IEF gels are not actually "focused" to their p*I*s. The separation is based on the migration rates of the differentially charged polypeptides as they move across the gel. Therefore, it is necessary to pay strict attention to accumulated volt-hours during the run to assure reproducible patterns in subse-

quent separations. During the prerun, a pH gradient is set up, and the focusing voltage is reached. The resistance in the gels is such that this voltage should be achieved in <2 h. If it takes longer, the samples may have high conductivity or the ampholyte quality may be inferior (*see* **Notes 4** and **7** in Chapter 15).

4. The conditions for optimum polypeptide separation will most probably have to be empirically determined for the NEPHGE gels. This is because the proteins are not focused to their respective p*I*s as in standard IEF. Therefore, a series of test runs with different accumulated volt-hours should be compared to optimize for each sample.

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17

High-Resolution, 2-D Protein Electrophoresis Using Nondedicated Equipment

Marion Sarmiento

1. Introduction

Several formats—small, standard, and large—can be employed to separate proteins by two-dimensional (2-D) protein electrophoresis. The number of proteins in the sample as well as the degree of resolution needed determine the particular format used. The small format is preferred when few proteins (usually <30 proteins that differ to a moderate extent in size and charge) are to be separated. This format allows for a significantly shortened analysis time. The small gels also are easier to handle than larger ones. Large formats are useful for separating large numbers of proteins (usually >200), many of which may have similar molecular sizes or charges. Large formats provide the highest degree of separating power of all the formats, although some resolution with respect to spot intensity may be lost in some areas of the gel. The standard format is the most commonly employed format, because it provides a significantly higher degree of separating power than the small format and improved spot intensity over the large format. The gels also are much easier to handle than the more cumbersome and fragile large format gels.

Although 2-D protein electrophoresis has been recognized for over two decades as one of the most powerful methods for separating proteins, its application in practice has been greatly hampered by the requirement for specialized, dedicated equipment as well as a high degree of skill in executing the technique. The expense for this specialized equipment (particularly for standard and large-format analyses) and for highly trained research technologists has been prohibitive for most laboratories.

This chapter describes a method for high-resolution, 2-D electrophoresis on a standard format that utilizes nondedicated electrophoretic equipment. Key fea-

tures of various well-known protocols are combined in a novel manner to yield an improved approach that can be employed by technologists experienced only in basic SDS-PAGE.

2. Materials

2.1. Nondedicated Equipment for the First Dimension (see Note 1)

1. Hoefer tube gel apparatus with 3-mm id grommets (Model GT-UB3-G; Hoefer Instruments, San Francisco, CA).
2. IEF gel-forming chamber—an acrylic tube (od, 1.3 cm; id, 1 cm; length, 12 cm).
3. 100 µL disposable glass micropipet (od, 2 mm; id, 1.5 mm; length, 12.7 cm; Clay Adams, Parsippany, NJ).
4. Acrylic rods (12 cm × 3 mm).
5. 0.22-µm Syringe filter.
6. Hoefer gel slicer (Model DE113 9; Hoefer Instruments, San Francisco, CA).
7. Beckman microelectrode for pH determination (Model 39535; Beckman Instruments, Palo Alto, CA).

2.2. Nondedicated Equipment for the Second Dimension

1. Hoefer slab-gel apparatus (*see* **Note 2**).
2. Glass plates (*see* **Note 3**).
3. Spacers of 1-mm thickness (SE6119-2-1.0, Hoefer Instruments).
4. Reference comb (SE511-R-1.0, Hoefer Instruments).
5. Small Teflon comb (SE211A-R-75; Hoefer, Instruments).

2.3. Reagents (see Note 4)

1. Piperazine diacrylamide (PDA) crosslinker (Bio-Rad, Hercules, CA; *see* **Note 5**).
2. Prestained SDS-PAGE mol-wt markers (Bio-Rad).

2.4. Solutions for First-Dimension IEF

1. 30% (w/v) Acrylamide (stored at 4°C).
2. IEF gel solution: 9 M urea; 4% (w/v) acrylamide; 0.24% (w/v) PDA; 0.8% (v/v) Triton X-100; 0.5-2.0% (w/v) ampholytes. Briefly, to make 10 mL IEF gel solution, 5.5 g urea crystals are mixed with 1.33 mL 30% w/v acrylamide, 3.83 mL H_2O, 0.33 mL carrier ampholytes, pH 3.0–10.0, 0.1 mL carrier ampholytes, pH 5.0–7.0, and 0.1 mL carrier ampholytes, pH 2.0–4.0 (*see* **Note 6**). The mixture is swirled gently in a water bath set at 37°C until the urea crystals are completely dissolved. Then, 0.024 g of PDA is added and dissolved (*see* **Note 7**). Excess dissolved gases are removed from the solution by placing under vacuum for a few minutes. Then, 0.4 mL 20% v/v Triton X-100 is added and gently mixed (*see* **Note 7**). The solution is passed through a 0.22-µm syringe filter to remove residual dust or microscopic particle impurities. This solution should be made fresh each time.
3. 10% (w/v) Ammonium persulfate (prepared fresh each time).

4. Urea solubilization buffer for IEF: 9 M urea, 4% (v/v) Triton X-100, 2% (w/v) ampholytes pH 9.0–11.0, 2% (v/v) 2-mercaptoethanol (stored at –20°C).
5. 20% (v/v) Triton X-100.
6. 10 *M* NaOH.

2.5. Solutions for Second-Dimension SDS-PAGE

1. 40% (w/v) Acrylamide (stored at 4°C).
2. Solution C: 5% (w/v) *N,N*'-diallytartardiamide (DATD) (Serva Biochemicals, Westbury, NY) in 40% (w/v) acrylamide (prepare fresh each time).
3. 1 *M* Tris-HCl, pH 7.0.
4. 0.06% (w/v) Bromophenol blue (BPB).
5. 50% (w/v) Sucrose (stored at 4°C).
6. 20% (w/v) SDS.
7. 0.52% (w/v) Ammonium persulfate (prepare fresh each time).
8. Separating gel solution: 1.6% (w/v) DATD; 12.7% (w/v) acrylamide; 3 *M* Tris-HCl, pH 8.5. Briefly, in a side-arm flask (250 mL) add: 9.3 mL solution C, 15.1 mL H$_2$O, and 4.9 mL 3 *M* Tris-HCl, pH 8.5. After swirling gently to mix, the mixture is degassed under vacuum just until bubbles began to form along the side of the flask. Make fresh each time.
9. Stacker gel solution: 1.5% (w/v) DATD; 12% (w/v) acrylamide; 0.2% (w/v) SDS; 0.25 *M* Tris-HCl, pH 7.0. In an Erlenmeyer flask (100 mL) add: 3.6 mL solution C, 5.3 mL H$_2$O, 3 mL 1 *M* Tris-HCl, pH 7.0, and 0.12 mL 20% (w/v) SDS. Make fresh each time.
10. SDS-PAGE buffer: 0.19 *M* glycine; 0.02 *M* Trizma Base. Mix 12 g Trizma Base and 57.6 g glycine in 4 L with H$_2$O. No SDS is used in the electrophoresis buffer.
11. SDS sample buffer: 0.05 *M* Tris-HCl, pH 7.0; 0.0018% (w/v) BPB; 1.25% (w/v) sucrose; 2% (w/v) SDS; 0.05% (v/v) 2-mercaptoethanol. Briefly, mix together 1 mL 1 *M* Tris, pH 7.0, 0.5 mL 0.06% BPB, 0.5 mL 50% sucrose, 2.0 mL 20% SDS, 1.0 mL 2-mercaptoethanol, and 15 mL H$_2$O. Store stock solution at –20°C.

3. Methods
3.1. IEF Dimension
3.1.1. Preparation of IEF Gels

1. Seal the IEF gel-forming chamber at one end with two layers of Parafilm and place upright in a rack.
2. Insert 22 100-µL disposable glass micropipets into the chamber (*see* **Note 8**).
3. Add 46.6 µL 10% w/v ammonium persulfate and 6 µL TEMED to the IEF gel solution, and gently mix.
4. Using a Pasteur pipet, transfer the IEF gel solution to the IEF gel-forming chamber. Fill the micropipets (by capillary action) from the bottom upward to approx 1 cm from their tops (*see* **Note 9**). Invert a 50-mL disposable plastic centrifuge tube over the IEF gel-forming chamber to prevent desiccation and precipitation of urea during polymerization. Allow the gels to polymerize for a minimum of 2 h. During this time, prepare the IEF samples (*see* **Note 10**).

5. Following polymerization of the IEF gels, remove the Parafilm, and push the micropipets out through the bottom of the plastic tube and into a tray containing deionized water. Gently separate the micropipets from each other, clean off external polyacrylamide, and immediately place into a tray containing a moistened paper towel.

3.1.2. Loading IEF Samples and Conditions for IEF Analysis

1. Fill the lower chamber of the Hoefer GT-10 tube gel apparatus with 2.5 L of degassed, deionized water to which 2.5 mL of 85% phosphoric acid has been added.
2. Assemble the IEF gel-containing micropipets into the IEF sample chambers (*see* **Note 11**).
3. After positioning the top chamber of the tube gel apparatus, insert the IEF sample chamber micropipet assemblies (*see* **Fig. 1**) snugly into the grommets.
4. Fill the top chamber with 500 mL of degassed, deionized water to which 1 mL of 10 *M* NaOH has been added.
5. Remove air from each sample chamber by gentle manual suction using a Pasteur pipet. Residual air present above each IEF gel is gently dislodged with the aid of a 23-gage spinal tap needle connected to a tuberculin (1-cc) syringe (*see* **Fig. 1**).
6. Apply the samples (10–15 μl) to the tops of the IEF gels (by gravity) with the aid of a Hamilton (100 μL) gas-tight syringe (*see* **Note 12**). For the experiments described in this study, no overlay solution is used, and the gels are not subjected to prefocusing.
7. The gels are focused at 400 V for 16–20 h (i.e., overnight), and then at 800 V for 30 min. Temperature is maintained at 25°C during IEF focusing with the aid of circulating, cooled water.
8. To minimize protein diffusion along the pH gradient axis, the micropipet gel tubes are removed from the sample chamber assemblies immediately after focusing and chilled. This is accomplished by placing the glass micropipets into test tubes (16 × 150 mm) immersed in ice.
9. The gels are either processed immediately for pH gradient determination or loaded directly onto SDS-PAGE gels within 2 h after IEF.

3.1.3. Processing IEF Gels for pH Gradient Determination

1. To process a gel for pH gradient determination, connect a 3-mL syringe containing electrophoresis buffer to a plastic disposable micropipet tip (trimmed with a razor blade to fit snugly onto the end of the syringe barrel; *see* **Figs. 1** and **2**).
2. Insert the tip into the top end of a glass micropipet containing an IEF gel and slowly extrude the gel onto a Parafilm sheet by gently forcing electrophoresis buffer into the micropipet. Similar methods for extruding IEF gels have been described previously *(1,2)*.
3. After aligning the gel appropriately, slice the gel into 0.5-cm sections with the aid of a razor-blade gel slicer. Using forceps, immediately transfer each gel slice
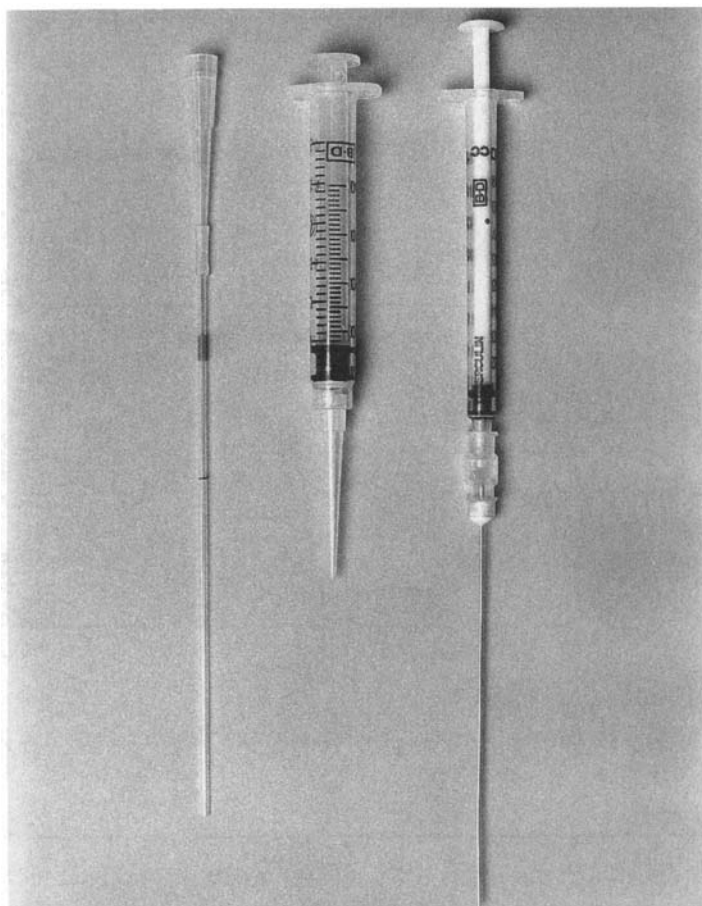
Fig. 1. Small instruments used in 2-D analysis. Left: Conical-shaped IEF sample chamber connected to the top of a glass IEF micropipet by a piece of silicone rubber tubing. Center: a disposable micropipet tip fitted onto the end of a 3-mL syringe (used for extruding the IEF gel from the glass micropipet). Right: a spinal tap needle connected to a tuberculin (1-mL) syringe (used to dislodge small air bubbles from the tops of IEF gels). Reprinted by permission from Sarmiento *(11)*.

     to a test tube containing 1 mL deionized water. Gently rock the samples for 4 h at room temperature.

4. After removing the gel slices, take pH measurements using a microelectrode (*see* Chapter 30).
5. To align the pH gradient properly with individual proteins, samples are vacuum-desiccated, dissolved in a small amount of SDS-sample buffer, and analyzed by conventional SDS-PAGE.
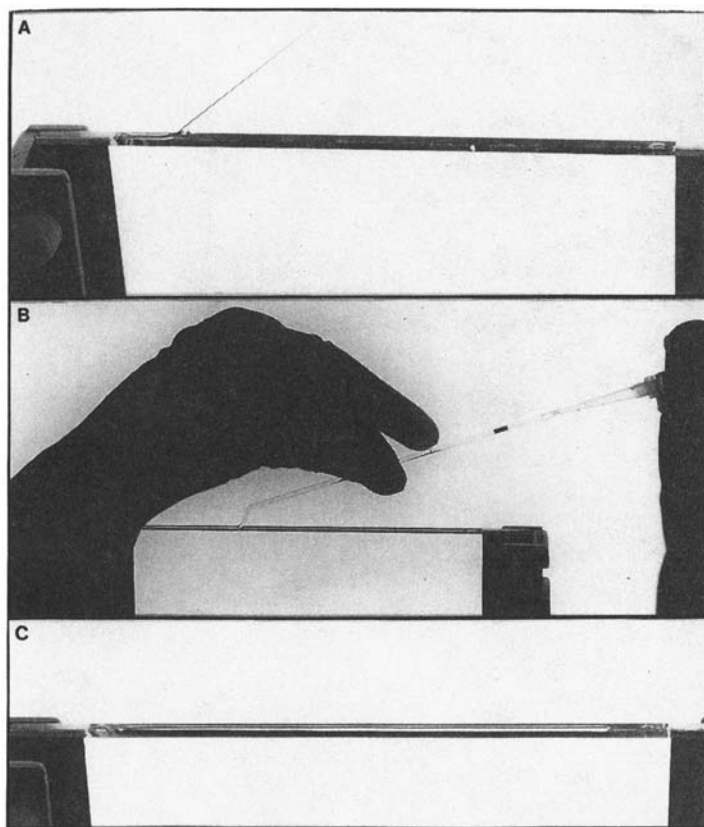
Fig. 2. Extrusion of first-dimension IEF gel onto the top of second-dimension SDS-PAGE gel. **(A)** Close-up of tip of IEF gel as it is gradually being extruded and laid on top of the groove between the glass plates forming the SDS-PAGE gel. **(B)** Detail of how the IEF gel is extruded by gently forcing SDS-PAGE buffer through the top end of the 100-μL glass micropipet using a micropipet tip attached to a 3-mL syringe. **(C)** The IEF gel is completely extruded and ready to be gently pushed down into the groove between the glass plates and onto the top of the stacker gel. Reprinted by permission from Sarmiento *(11)*.

3.2. Second-Dimension SDS-PAGE

3.2.1. Preparation of SDS-PAGE Gels

The method of SDS-PAGE analysis used in this study is based on one originally described by Laemmli *(3)* and later modified by Morse et al. *(4)*. The method has been further altered so that the ratio of acrylamide to DATD crosslinker is 8:1 (w/w). The higher DATD content gives more strength to the

gels and reduces stretching during staining procedures. In addition, SDS is omitted from the electrophoresis buffer. The SDS present in the stacker gel and in the SDS sample buffer is sufficient to maintain proteins properly solubilized during the analysis. Gels of uniform acrylamide content (% T = 9.5) are used. Other applications employing gradients of acrylamide or different crosslinkers have been described *(5,6)*.

1. Following assembly of the glass plates onto the SDS-PAGE gel rig, mix the separating gel solution, and pour as follows (per two gels).
2. Add 9.7 µL TEMED and 9.7 mL 0.52% ammonium persulfate to the separating gel solution, and mix gently (but thoroughly) by swirling.
3. Using a 10-mL pipet with an elongated tip, add 18.5 mL to each chamber by gently pouring down the side (*see* **Note 13**). Immediately overlay the gel solution with 0.5 cm of H_2O using a 23-gage needle attached to a 5-mL syringe (without the plunger) containing 2 mL H_2O. Allow the water to slide down the side of the chamber gently by gravity (*see* **Note 14**). Allow the gels to polymerize for at least 1 h (*see* **Note 15**).
4. Add 5 µL TEMED and 12 µL 0.52% ammonium persulfate to the stacker gel solution, and gently swirl to mix.
5. Carefully decant the water overlay from the tops of the (polymerized) separating gels, and rinse the chamber above each gel three times with 1- to 2-mL stacker gel solution.
6. After filling each chamber with stacker gel solution, insert a reference comb (*see* **Note 16**) to form the IEF gel sample and reference wells at 2 cm above the separating gel. If a notched divider plate (*see* **Note 2**) is used to cast two gels together, any stacker solution that overflows above the top of the divider plate after insertion of the comb is removed to prevent formation of an acrylamide collar on top of the glass plates. Allow the stacker gel to polymerize for 20–30 min.
7. Following polymerization, flood the top of each gel with electrophoresis buffer so that as the reference comb is gently removed, the wells formed by the comb fill with buffer rather than with air.
8. Fill the wells to the brim with electrophoresis buffer, and immediately load the IEF gels.

3.2.2. Loading SDS-PAGE Samples and Conditions for SDS-PAGE Analysis

Equilibration of IEF gels is accomplished at the time of loading the gels onto the 2-D SDS-PAGE gels. No special equilibration buffer was required for this step.

1. Slowly extrude the gel by gently forcing electrophoresis buffer into the glass micropipet using a 3-mL syringe connected to a polypropylene micropipet (*see* **Figs. 1** and **2**) as described above. As the gel slips out of the glass micropipet, lay the gel evenly and without stretching onto the top of the groove between the two glass plates forming the SDS-PAGE gel (**Fig. 2**).

2. Using the back of a small Teflon comb (0.75 mm in thickness), gently and evenly push the IEF gel downward between the glass plates (taking care not to trap air bubbles) until the gel is in direct contact with the top of the stacker gel. During this procedure, sufficient urea and carrier ampholytes elute from the gel to equilibrate the gel with the electrophoresis buffer.

3. Remove the loading electrophoresis buffer from the sample well (to wash away any debris as well as eluted urea and carrier ampholytes that may interfere with SDS-PAGE) with the aid of a 23-gage spinal tap needle connected to a tuberculin syringe.

4. Load other gels to be run simultaneously on the same apparatus at this time.

5. Fill the sample and reference wells to the brim with fresh electrophoresis buffer.

6. Place prestained mol-wt marker by gravity (5 μL; Bio-Rad) and SDS sample buffer (0.2 mL) into the reference and sample wells, respectively.

7. Immediately insert the SDS-PAGE gels into the electrophoresis chamber, and perform electrophoresis at 30 mA/gel for 3 h or until the BPB blue dye front is within 1.5 cm of the distal end of the gel. The gels are maintained at 20°C during electrophoresis with the aid of circulating cooled water.

8. Following electrophoresis, place the plates containing each gel on ice (to minimize diffusion) until the plates can be removed and the protein spots in the gels either stained directly (*see* **Note 17**), transferred by PVDF membrane by Western blot method, or detected by autoradiography (*see* **Note 18**).

3.2.3. Resolution and Reproducibility of Method

Figure 3 depicts typical results of 2-D electrophoresis analysis obtained by the described method. The extremely high reproducibility of the method is evident by the comparison of two typical analyses, shown in **Fig. 3A** and **C**, that were conducted at a different time than those depicted in **Fig. 3B** and **D**. Comparison of the protein patterns from cells that were either untreated (**Fig. 3A** and **B**) or treated (**Fig. 3C** and **D**) with interferons also reveals that the method is highly reproducible and sufficiently powerful to the analyze samples that have differences in only a few proteins. The protein patterns are sharp and clearly visible in both analyses. Differences in protein expression between untreated and treated cells are readily discernible. Proteins showing little or no change in expression are located in almost identical positions between various analyses, elucidating the reproducibility and uniformity of the method. To provide a reference point, the position of the major (58-kDa) protein known to be upregulated by IFN-γ in fibroblasts, tryptophanyl-tRNA synthetase (TrpRS) *(7,8)*, was identified by horseradish peroxidase-based immunoblot assay (Vectastain ABC, Vector, Burlingame, CA), using specific primary antibodies, and is indicated in **Fig. 3C** and **D**. Two other unidentified proteins that are markedly upregulated by IFN treatment are also indicated.
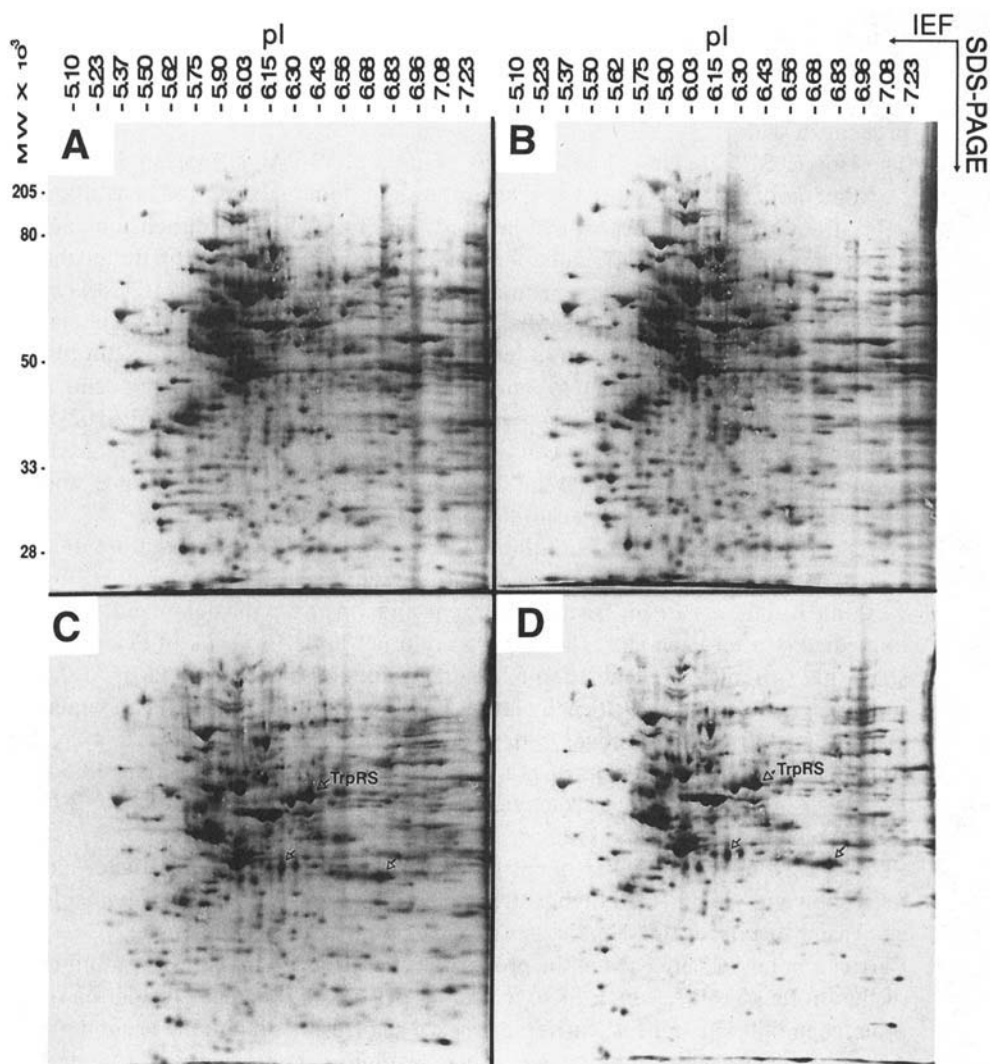
Fig. 3. 2-D analyses of proteins from WISH cells that were either untreated (panels **A** and **B**) or treated with conIFN-α and IFN-γ (panels B and **C**) for 16 h before labeling with L-[^{35}S]methionine for 1 h. Approximately 20×10^6 dpm of sample were used/analysis. The separated proteins were transferred to PVDF membrane and detected by autoradiography for 12–24 h. The position of TrpRS and of other major proteins induced by IFN-γ are indicated. Reprinted by permission from Sarmiento *(11)*.

4. Notes

1. The use of disposable latex gloves is strongly recommended throughout all procedures. The gloves are washed on the outside surface (while being worn) to

remove powder or other contaminants that may interfere with the analyses. It is critically important to use deionized water (conductivity >18 MΩ) for making all solutions and buffers to minimize interference by impurities present in water.

2. The Hoefer SE600 slab-gel apparatus is used for SDS-PAGE analysis (Hoefer Instruments). This or a similar unit that allows building stacker and separating gels of equivalent dimensions can be used. The following gel dimensions are critical for the proper accommodation and appropriate "snugness-of-fit" of the first-dimension IEF gels: The separating gels should be 1 mm in thickness, 14 cm in width, and at least 16 cm in length. The unit should allow for a stacker gel that is 14 cm wide and at least 2 cm in length. The Hoefer SE600 normally can run one or two gels at one time; up to four gels can be run simultaneously by using a notched glass divider plate to cast two gels on each side of the unit (SE6102D, Hoefer Instruments). This unit can also be adapted for analyzing SDS-PAGE gels of any length by modifying the lower buffer chamber, the glass plates, and the spacers to accommodate gels of the desired length.

3. Glass plates should be cleaned thoroughly (preferably acid-washed) before they are used for the first time. The plates can be kept clean between analyses by washing in laboratory glassware detergent and rinsing with deionized water immediately after each use. The plates should not be left to soak in detergent, since this promotes the deposition of detergent film on the glass surface. At the time of assembly, the side of each plate that will contact the gel should be wiped clean of any remaining residues with 95% ethanol.

4. Chemicals of the highest reagent-grade purity are used throughout this method. Wherever possible, chemicals recommended for isoelectric focusing or for 2-D protein electrophoresis are used.

5. PDA is used as a crosslinker in casting IEF gels, because this crosslinker has been shown to yield gels of higher strength and porosity, which are more pliable and easier to extrude and handle than gels cast with *bis*-acrylamide *(5)*.

6. Carrier ampholytes are used in the proportions described to maximize resolution of the protein TrpRS, which focuses around pH 6.4. To increase resolution of proteins in other pH ranges, carrier ampholytes corresponding to the desired pH range can be used (for example, to optimize resolution within or near the neutral pH range, the volume of carrier ampholytes pH 5.0–7.0 can be increased to 0.2 mL and the carrier ampholytes pH 2.0–4.0 can be omitted).

7. Addition of PDA to the solution before the urea crystals are completely dissolved results in clumping and interferes with the dissolution of the PDA. The solution should not be allowed to boil during the degassing procedure. Nonionic detergent, such as Triton X-100, must be added after degassing to prevent reduction of the solution surface tension and decreasing the boiling point during degassing. A 20% v/v Triton X-100 solution is significantly easier to dispense and dissolves more efficiently than 100% Triton X-100.

8. Acrylic rods are inserted, as needed, to displace and conserve IEF gel solution when fewer than 20 gels were cast.

9. This corresponds to the approximate height necessary to create IEF gels (11.7 cm in length) that would fit across the tops of the slab gels used in these studies. The capillary action filling method of casting also yields IEF gels of uniform length.

10. The methodology described details the 2-D analysis of proteins from eukaryotic cells grown in tissue culture. For these experiments, WISH cells (human amniotic fibroblasts, American Type Culture Collection, Rockville, MD) were grown to confluency at 37°C in 150-cm^2 flasks (2.4×10^7 cells/flask) with 30 mL of RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum. To illustrate further the reproducibility of the methodology, some of the cells were stimulated with a combination of conIFN-α and IFN-γ (10 ng/mL each) for 16 h at 37°C to induce the expression of certain proteins *(9,10)*. The cells were then labeled with L-[^{35}S]methionine (10 μCi/mL) for 1 h at 37°C, harvested by scraping into cold PBS, collected by centrifugation, and washed twice with cold PBS. Pellets containing 1×10^7 cells were either used immediately or were frozen at –80°C promptly after removing the supernatant. No change in protein pattern or resolution was observed in cells stored frozen at –80°C for several months. Briefly, 150 μL of urea solubilization buffer for IEF were added directly to each fresh or frozen cell pellet, followed immediately by rapid pipeting. It was important not to thaw the cell pellets prior to addition of the buffer to prevent the release and activation of endogenous proteases. The samples were rocked gently at room temperature for at least 2 h. DNA and other insoluble material was removed by centrifugation at 175,000*g* for 1 h at 24°C (i.e., 70,000 rpm in the Beckman TLA-100 rotor, Beckman Instruments, Palo Alto, CA). The supernatant was transferred to a conical polypropylene microtube (taking care not to collect any pelleted material) and held at room temperature until loaded onto the IEF gels. Samples were analyzed on the same day that they were solubilized. The solubilized samples were not refrozen, because this introduced artifacts that affected the migration of proteins, particularly in the IEF dimension.

11. To assemble conical-shaped IEF sample chambers, polypropylene micropipet tips (100–200 μL volume) are cut with a razor blade to create a small tip end with an outside diameter of 2 mm (the od of the IEF glass micropipet). A 1-cm segment of silicone rubber tubing of od 3/32 in. and id 1/32 in. (#6411-60 Cole Parmer Instrument, Chicago, IL) is connected to the small end of each polypropylene micropipet tip. The open end of the silicone rubber segment is then connected to top of an IEF gel-containing micropipet (taking care to align into flush contact the end of the polypropylene micropipet tip with the top of the glass micropipet. The conical shape of the polypropylene micropipet tips allows a snug fit into the grommets in the upper buffer chamber and, in addition, facilitates sample loading. These IEF sample chambers are reusable.

12. This amount of sample is equivalent to 0.6×10^6–1×10^6 cells/IEF gel. It is important not to exceed 1×10^6 cell equivalents/gel to prevent trapping protein complexes at the top of the gel and consequent loss in IEF resolution.

13. It is important not to pour the separating gel solution down the center of the plates, because traces of gel solution remaining on the plates above the top of the separating gel would dry during the polymerization period and would interfere with the resolution of the SDS-PAGE protein separation.

14. To minimize splashing, it is best to pour H_2O down the same side that was used to pour the gel solution.

15. At least 1 h at room temperature is necessary to polymerize fully and cure the DATD-acrylamide gels. Movement of the gels before this time results in the production of a double meniscus at the top of the gel. This interferes with the resolution of the SDS-PAGE protein separation.

16. It is important not to trap any air bubbles beneath the reference comb. This is most easily accomplished by filling the chamber to the brim with stacker solution before inserting the comb. Excess solution is removed after comb insertion. Parafilm or plastic wrap is used to cover the top of the gel rig to prevent desiccation during polymerization.

17. To prevent expansion and distortion of the gels containing DATD acrylamide, solutions used to fix and destain proteins in these gels should not contain more than 5% (v/v) glacial acetic acid; the gels should not be left to soak in solutions containing acetic acid for extended periods.

18. It was found that radiolabeled proteins can be detected with 10-fold greater efficiency by autoradiography after transfer onto PVDF membranes than was possible in fixed, dried gels. (This is probably because the gel matrix quenches much of the energy emitted by radioisotopes, such as ^{35}S; energy emitted from L-[^{35}S]methionine labeled proteins that have been transferred out of the gel matrix and onto the surface of a PVDF membrane reaches the autoradiographic film more efficiently.)

Acknowledgments

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18

Large-Gel 2-D Electrophoresis

Joachim Klose

1. Introduction

Two-dimensional electrophoresis (2-DE) of proteins is used for several purposes, such as resolving a distinct group of proteins (e.g., serum proteins), revealing the heterogeneity of a particular protein (e.g., actin, transferrin), purifying a protein, or testing the purity of a protein gained by other methods. However, the most exceptional feature of this method is its potentiality to resolve all the various proteins of a certain cell type or tissue. It was this particular feature of the 2-DE technique developed in 1975 *(1,2)* that opened the way to study many of the biological problems of present interest under a new aspect: Problems of gene expression, gene regulation, genetic variation, cell differentiation, embryonic development, pathogenesis of certain diseases, and other fields could be studied on the basis of a broad spectrum and a representative number of proteins, whereas previously studies like these were performed on selected proteins, often selected because of their easy accessibility and considered as model proteins (e.g. hemoglobin). Moreover, when simple procedures for extracting cell or tissue proteins were used, i.e., procedures that avoid steps, such as protein precipitation, lyophilization, dialyses, or chromatographic fractionations, 2-DE protein patterns revealed the individual proteins in quantities that reflect, at least to some extent, the relative concentration of these proteins in the cells or tissues. Quantitative data on gene expression offer an important parameter for all studies in cell biology. Furthermore, once the proteins are separated by 2-DE, adapted protein analytical techniques, such as blotting, partial sequencing, and peptide analysis by mass spectrometry, allow the characterization of the individual proteins and the identification of their genes. While, in a worldwide effort, sequencing of the whole human genome

(and the genome of other organisms) proceeds, 2-DE becomes an indispensable tool in the elucidation of the function of all the genes.

Is it possible to reveal all the various protein species of a single cell type by 2-DE? If 2-DE is able to reveal actually each individual protein of a particular cell type, this method would reach a new level of significance in studying biological and pathological processes. Because there is probably no process in a living cell that does not alter the occurrence or concentration of at least one protein, it would be worth for any study in basic and applied biomedical research to include a look at the 2-D protein patterns relevant for the problem under investigation. If, however, there is no guarantee that even the rarest transcription factor can be detected in these patterns, the information expected cannot be considered to be complete, even if it is complete.

The best present estimation of the total number of genes (defined as a region of DNA sequences that is transcribed into polyadenylated mRNA that is translated into protein) in the human genome is 60,000–70,000 *(3)*. Based on RNA reassociation kinetics, a typical mammalian cell may express 10,000 distinct genes *(4)*. We estimated from 2-DE protein patterns (soluble fraction of mouse brain proteins), which resulted from the large-gel technique described here, that, on the average, each individual protein is represented by 2.2 spots in the 2-DE pattern. This estimation was based on a genetic approach: Protein spots that showed in the 2-DE pattern of one mouse strain compared to another strain a shift in the position by exactly the same distance (e.g., 2.5 mm) and exactly in the same direction and, moreover, mapped to the same locus on the mouse chromosomes, were considered as modifications of the same protein. Consequently, a 2-DE pattern that reveals all the various proteins of a typical mammalian cell should consist of 20,000–25,000 (10,000 × 2.2) protein spots. Theoretically, a 2-D gel with the dimensions of 400 × 300 mm would offer place for 30,000 spots when the mean size of a spot is defined by 2 × 2 mm. The probability that two protein species of a cell are completely identical with regard to their p*I*, molecular weight, and three-dimensional shape and, therefore, occupy exactly the same locus in the 2-DE gel is rather unlikely *(5)*. According to these considerations, large-gel 2-DE should be able to reveal all the various proteins of a single cell type.

Several problems argue against the conclusion put forward above. The cell proteins do not spread evenly over the whole gel area and streaking spots occupy additional space. Furthermore, proteins may become lost selectively on the way from the tissue to the protein sample and during migration into the gel. Most critical, however, is that certain classes of proteins may exist in very low concentrations in a tissue, i.e., only a few molecules of a protein occur per cell and possibly not even in each cell of that tissue *(6)*. Proteins involved in regulatory processes and characterized by high turnover rates may represent

such a class of proteins. If rare proteins are defined as proteins present in a tissue in concentrations of 1 molecule/cell or group of cells, we believe, according to an experimental approach described elsewhere *(7)*, that rare proteins cannot be detected by presently available 2-DE techniques. However, defining the concentration of rare proteins as 3–30 molecules/cell, Duncan and McConkey *(8)* conclude from their investigations that rare proteins can be detected by 2-DE. Fractionation of tissue proteins followed by concentrating the fractions and running each fraction separately may be a way to overcome both the problem of restricted place on a 2-D gel and the problem of the low abundant proteins. Selective loss of proteins during sample preparation and during incubation of the gel alter isoelectric focusing (IEF) can be avoided by following the sample preparation procedure described in Chapter 9 and by omitting the gel-incubation step. However, even under the conditions, which we recommend for sample preparation and 2-DE, there is no guarantee that even the rarest transcription factor can be detected.

Nevertheless, in using 2-DE, our aim is to resolve and detect all the individual proteins of a distinct tissue. Therefore, a special procedure for extracting total tissue proteins was developed (Chapter 9) and our original 2-DE technique *(1,9)* was substantially altered under this aspect *(10)*. The main feature of the 2-DE technique described in the following is the use of large gels with a resolving power of more than 10,000 polypeptide spots/pattern (*see* **Note 15**). The protein sample is applied on the acid side of the IEF gel to avoid loss of the very basic proteins. On the acid side, the complete loss of certain protein species should scarcely occur, since very acid proteins (p*I* below 4) are rare. Immobilized pH gradients in comparison to carrier ampholyte gels were found to lose resolution, particularly in the basic range, when used in long-distance gels *(10)*. Moreover, when comparing 2-DE patterns of the same protein sample in the two different systems, immobilized pH-gradient gels retain more protein at the start position and, after Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), in the running gel than the carrier ampholyte gels (**ref. *11***; own observations). Therefore, if high resolution is the aim, we use carrier ampholytes and large gels.

The following describes the large-gel 2-D electrophoresis technique developed in our laboratory *(10)* in all detail. This includes the preparation of 40-cm IEF gels in capillary tubes, cutting the long gels into two halves after the IEF run, transfer of the two half-gels onto two normal-sized SDS gels with a running distance of 30 cm, silver staining of the separated proteins, and drying the 2-D gels. The slightly different conditions used when IEF is performed in gels of normal size (20 cm) are mentioned. This modification may be employed for protein samples of lower complexity. Recipes are given for preparing the gel solutions in portions ready to freeze and to use later. Freezing large numbers of ready-made portions from the same batch of gel solution contributes consider-

ably to the reproducibility of the 2-DE patterns (*see* **Note 4**). Large-gel 2-DE techniques were also described by other investigators *(12)*.
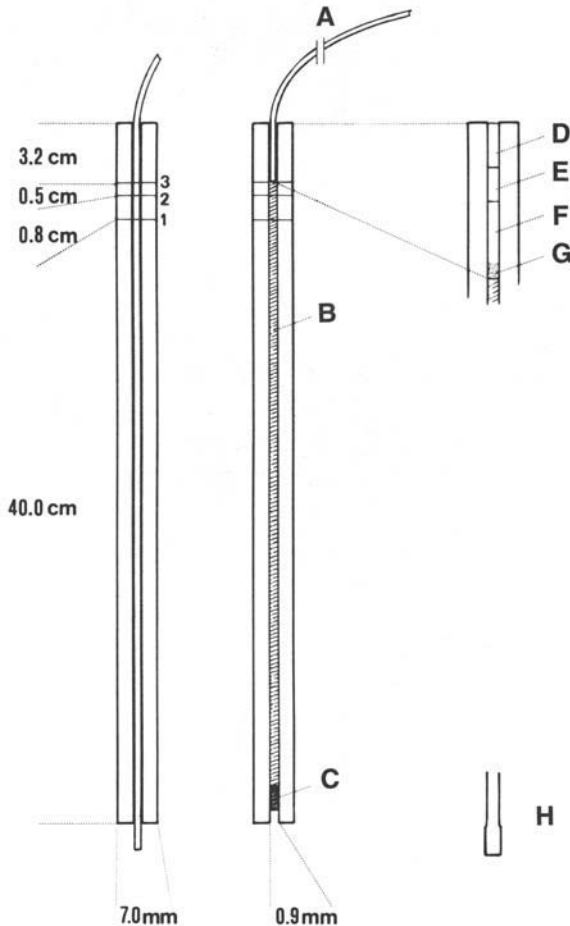
2. Materials

2.1. Equipment

2.1.1. IEF

1. Glass tubes for IEF gels: The dimensions of the glass tubes used for 40-cm gels and 20-cm gels are given in **Fig. 1** (*see* **Note 6**). To fill the tubes with gel solution and to push the gels out of the tubes after IEF, nylon strings (fishing line) are used. The strings are 0.8 mm thick and one end is made thicker (0.9 mm; **Fig. 1H**) by melting the end of the string and fitting it quickly into the glass tube. (Glass tubes from Schott Glaswerke, D-55014 Mainz, Germany; ready-made glass tubes and strings are available from WITA, Potsdamer Str. 10, D-14513 Teltow, Germany.)

2. Gel-tube stand: This apparatus consisted of a tube holder and an adjustable platform (**Fig. 2**). A special gel-solution groove is placed on the adjustable platform to bring the gel solution in contact with the ends of the glass tubes (**Fig. 2**).

3. Apparatus for IEF: The apparatus consisted conventionally of an upper and a lower cylindrical buffer chamber, but is equipped with an intermediate cylinder, available in various heights, to allow the insertion of gel tubes of various lengths (44.5 or 23.5 cm; available from WITA). Maximally, eight glass tubes are inserted into the apparatus and fixed with screws.

4. Power supply: The power supply for IEF should be programmable and allow the user to adjust a maximum of at least 2000 V (e.g., Electrophoresis Power Supply—EPS 3500 XL; Pharmacia, D-79111 Freiburg, Germany).

5. To store the gels after IEF and to facilitate the transfer of the IEF gels to the SDS-PAGE gels, special gel grooves are made from Plexiglas (*see* **Fig. 3**; available from WITA) and a box to fix these grooves in an orderly way. A wire formed as shown in **Fig. 3** is used to manipulate the thin and long gels.

2.1.2. SDS-PAGE

1. Polymerization stand: The polymerization stand Desaphor VA from DESAGA (D-69009 Heidelberg, Germany) able to hold two gel cells is used. A hole is made in the middle of the bottom of the stand through the bottom and the silicone gasket. Insert a cannula into this hole in such a way that the opening is located exactly between the two glass plates of the gel cell, with its other end projecting

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Fig. 1. *(opposite page)* Schematic representation of glass tubes for IEF gels and strings to suck the gel solution into the tubes. Left side: The filling string is in the start position. Center: The gel solution is in the tube, and the filling string **(A)** is in the polymerization position. (The lower end of the string is slightly thickened; **H**.) The tubes were filled by dipping the end of the tubes into the gel solution (*see* **Fig. 2**) and lifting the string, first to marking 1 to suck in the separation gel solution **(B)**, then to marking 2 to suck in the cap gel solution **(C)**, and finally to marking 3 to obtain, at the end of the tube, a small space free of gel solution. At the beginning of this filling procedure, wet the end of string and tube with the gel solution by moving the end of the string several times out of the tube and into the tube. After the separation gel solution is filled in, the gel solution groove (**Fig. 2**) is removed to exchange the rest of the gel solution by cap gel solution. The residual solution at the outside of the tube ends is wiped off with filter paper after the first and second step. The marking of the glass tubes for 20-cm gels had the following distances: tube end to marking 1: 20.2 cm; marking 1–2: 0.8 cm; marking 2–3: 0.5 cm; marking 3 to tube end: 2.0 cm (total length 23.5 cm). Right side: The upper end of a gel tube is shown indicating the various layers of solution after sample application: phosphoric acid **(D)**; protection solution, 5 mm **(E)**; sample solution, 5–10 µL, maximum 16 µL **(F)**; Sephadex suspension, 2 mm **(G)**. Sample solution in 20-cm gel tubes: 5 µL, maximum 10 µL. Phosphoric acid was omitted in cases of maximum sample load.
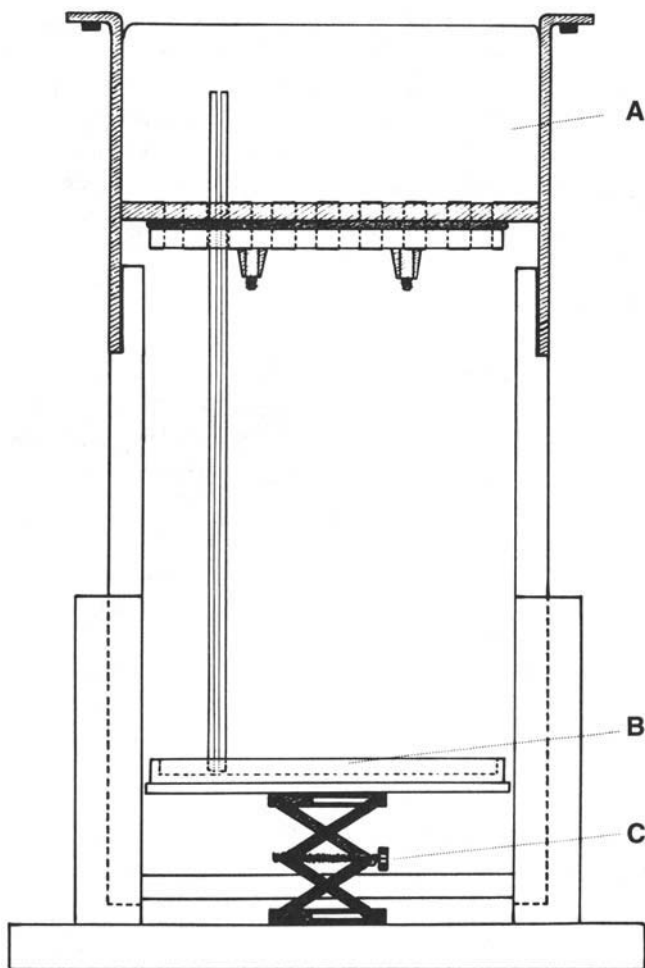
Fig. 2. Schematic representation of the gel-tube stand. Tube holder (**A**): The upper buffer chamber of the DESAPHOR VC 260 apparatus (DESAGA) was adapted to our gel-tube stand. Only 8 of the 24 holes of the real chamber are indicated in the scheme. Gel solution groove (**B**): The inner dimensions of the groove are 13 x 0.9 cm, deepest point of the curved bottom: 0.6 cm. Adjustable platform (**C**): used to move the gel solution up and down during gel preparation. An improved version of this apparatus is available from the WITA company.

   slightly from the bottom. Fix a fine tube to the free end of the cannula. Insert into this tube a 2-mL syringe.
2. To pour the gel solution into the glass cell, construct a special funnel (**Fig. 4**).
3. Apparatus for PAGE: The apparatus Desaphor VA 300 (DESAGA) is used. Two gel cells can be inserted into this apparatus. The measurements of the gel cells are indicated in **Fig. 4**. Plastic spacers, 0.75 mm thick, are used to fix the distance between the two glass plates of the cell.
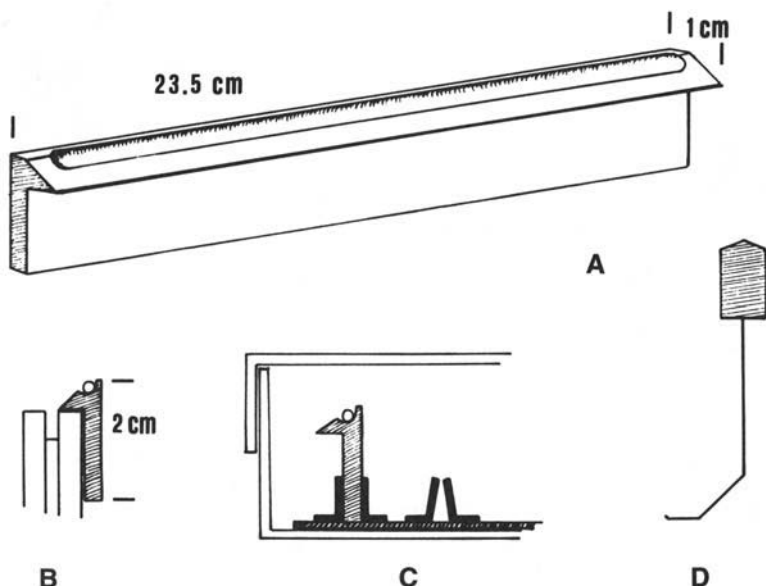
Fig. 3. Scheme of the gel groove. (**A**) Total view. (**B**) Cross section of the groove hold-ing an IEF gel. The groove is adapted to the gel cell, demonstrating the situation of IEF gel having to be transferred onto the SDS-PAGE gel. A wire suitably formed (**D**) is used to slide the gel between the glass plates. (**C**) To store the IEF gel in a freezer, the gel groove carrying the gel is fixed by a metal clamp on a plate and protected by a plastic box.

4. Power supply: The power supply for PAGE should be equipped with a capacity of at least 100 mA and 1000 V (e.g., *see* **Subheading 2.1.1.**).

2.1.3. Gel-Drying

Incubator: For drying up to eight 20×30-cm gels, the incubator type UL-60 from Memmert (D-91126 Schwabach, Germany) is used in a modified version: holes are made through the two side walls of the incubator on four different levels (**Fig. 5**), a modification carried out by Memmert on request. Drying plates serving as supports for the gels are manufactured from aluminum in a workshop and are described in **Fig. 5**. Eight plates are connected by a tube system that pass through a manometer, one on the left and one on the right side, and end in a water-saving vacuum pump, type TOM JET-1/A4 (Genser Wissenschaftliche Apparate, D-91541 Rothenburg, Germany; **Fig. 5**). For the drying procedure, various sheets including the gel are layered onto the drying plate (**Fig. 5**): V4A steel plate perforated with 0.93-mm holes at a distance of 1.9 mm between the holes, ordered from a metal ware shop; filter paper type 0859 (filtration time 150 sec) from Schleicher and Schuell (Keene, NH 03431); water-permeable clear membrane, the cellophane membrane backing from Bio-
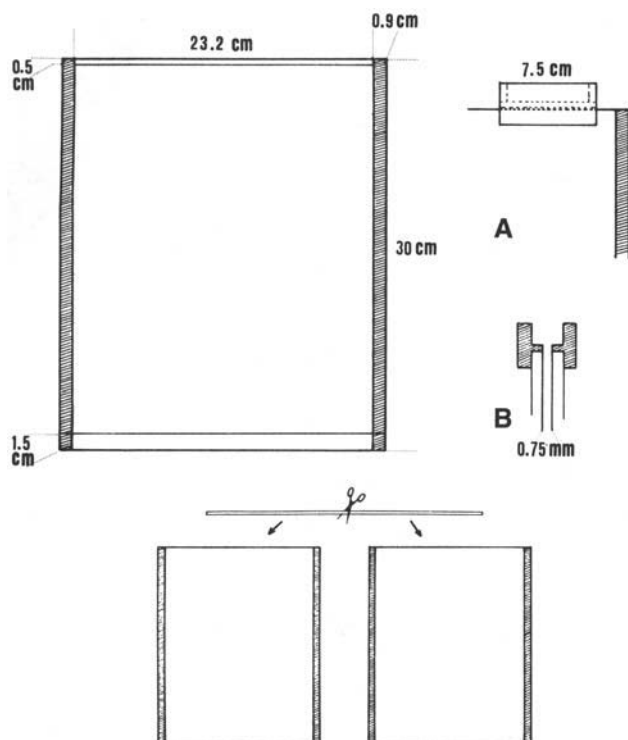
Fig. 4. The gel cell (without clamps) of the electrophoresis apparatus DESAPHOR VA 300 (DESAGA) is shown schematically. The horizontal line below the upper edge of the glass plate indicates the surface level of the separation gel. The horizontal line above the lower edge of the glass plate was scratched into the glass as a marking for finishing the electrophoretic run. The hatched stripes on each side of the gel cell indicate the plastic spacers between the two glass plates. A special plastic funnel to pour the gel solution between the glass plates is shown in frontal view (**A**) and in a cross section (**B**). The lower part of this figure depicts a situation in which the IEF gel is cut into two halves to transfer the 40-cm tube gel to two regular-sized SDS-PAGE gels.

Rad Laboratories (D-80901 München, Germany); porous polyethylene sheet from Hoefer Scientific Instruments (San Francisco, CA 94107); silicon sheet; aluminum plate with a coin-sized hole in each corner (to allow penetration of air) and equipped with a handhold.

2.2. Buffers and Solutions (see Note 5)

2.2.1. Isoelectric Focusing (IEF)

1. Gel solution: The composition is given in **Table 1**. The gel solution is filtered (GF/C glass microfiber filter, diameter 7 cm, from Whatman Scientific Ltd.,
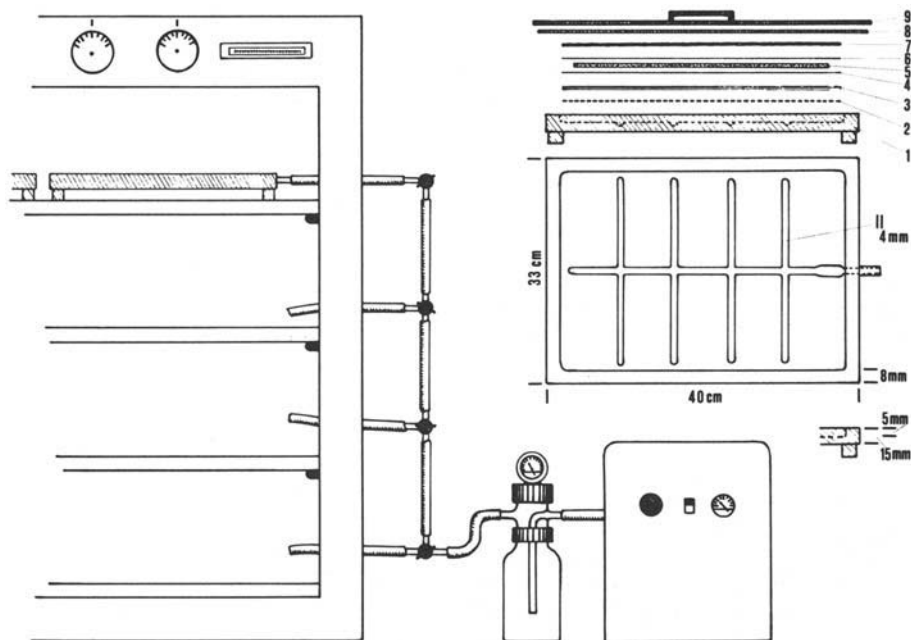
Fig. 5. Scheme of the gel drying system. The incubator (only the right half is shown) with inner dimensions of 100 × 80 (height) × 45 cm (depth) has four levels, each bearing two drying plates. One plate and part of the second one are shown on the fourth level. The four plates of one side are connected by a tube system that ends in a water-saving water vacuum pump. A manometer combined with a water trap is integrated in the tube system. Circuit breakers on each level of the tube system allow the generation of a vacuum for each plate individually. The vacuum pump is connected with the tube system of both the left side (not shown) and the right side. A drying plate is shown from above and from the front. A system of rills in the bottom of the plate drains the water sucked off under vacuum from the wet sheets layered on the plates. The different layers are shown: (1) drying plate; (2) metal plate perforated with fine holes, 31.0 × 37.8 cm, 0.7 cm thick; (3) 10 layers of filter paper; (4) water-permeable clear membrane; (5) SDS-PAGE gel; (6) water-permeable clear membrane; (7) porous polyethylene sheet; (8) silicon sheet, 44 × 37 cm, 1 mm thick; (9) metal plate, 45 × 36 cm, 3 mm thick. When the vacuum pump is turned on, the silicon sheet is pressed with the metal plate against the drying plate until a vacuum is generated in the space between silicon sheet and drying plate.

    Maidstone, Kent, ME 160LS, UK), aliquoted into 0.975-mL portions, and stored at –70°C.
2. Cap gel solution: The composition is the same as that of the gel solution (**Table 1**) with the exception of the acrylamide stock solution, which is replaced by the following solution: 12.0 g acrylamide and 0.13 g piperazine diacrylamide in

Table 1
Gel Solution for IEF Gels

Components	Stock solutions[a]	Mixture	Final concentrations
Acrylamide	3.5 g		3.50%
Piperazine diacrylamide	0.3 g		0.30%
	in 20 mL[b]	10.00 mL	4.00%
Carrier ampholyte mixture	8.0 mL[c]	5.00 mL	
Urea	—	27.00 g	54.00% (9.0 M)
Glycerol	14.3 g/50 mL	8.75 mL	5.00%
TEMED	60.0 µL/10 mL	5.00 mL	
Gel solution		48.75 mL	0.06%

[a]The chemicals were dissolved in bidistilled water; the final volumes are given.

[b]Amberlite treatment of acrylamide solution: The 20-mL acylamide solution was mixed with 1 g Amberlite MB-1A and agitated for 1 h. Immediately thereafter the solution was filtered to remove Amberlite (Amberlite may alter the pH of the solution). Amberlite was washed several times with bidistilled water on a filter before use.

[c]The mixture of carrier ampholytes was a combination of 8 mL Ampholine, pH 3.5–10.0 (Pharmacia, D-79111, Freiburg, Germany), 8 mL Servalyt, pH 2.0–11.0 (Serva), 24 mL Pharmalyte pH 4.0–6.5 (Pharmacia), 16 mL Pharmalyte, pH 5.0–8.0, and 8 mL Pharmalyte, pH 6.5–9.0. The resulting 64-mL ampholyte mixture was aliquoted into 8-mL portions, frozen in liquid nitrogen, and stored at –70°C. One 8-mL portion was used for the gel solution and the cap gel solution and the rest was aliquoted into 60-µL portions, frozen, and used for the Sephadex suspension (*see* **Subheading 3.2., step 5**). The original ampholyte solutions were used immediately after supply. **Note:** Pharmalyte 6.5–9.0 is no longer available from Pharmacia; try Ampholine, pH 7.0–9.0 from Pharmacia or Resolyt, pH 6.0–9.5 from Merck.

bidistilled water to give a final volume of 20 mL. Only half the volume of the mixture indicated in **Table 1** is prepared and then filtered, aliquoted into 0.390-mL portions, and stored at –70°C.

3. Ammonium persulfate solution: 0.08 g ammonium persulfate is dissolved in bidistilled water to give a final volume of 10 mL. The solution was filtered, aliquoted into 100-µL portions, and stored at –70°C.

4. Sephadex suspension: 20 g Sephadex G-200 superfine is swollen in 500-mL bidistilled water for 5 h at 90°C. The supernatant water is decanted, and the Sephadex resuspended into 1 L 25% glycerol solution and stirred for 2 h using a propeller. The glycerol solution is changed once during the 2-h period. The supernatant water is then sucked off from the suspension by using a filter funnel. The pasty Sephadex is aliquoted into 8-mL portions and stored at –20°C. Prior to use, each portion is further aliquoted into 0.272-g portions and stored again at –20°C.

5. Sample protection solution: 6 g urea and 1 g glycerol are dissolved in bidistilled water to give a final volume of 19 mL. From this solution, 7.6 mL are mixed with 0.4 mL Servalyt, pH 2.0–4.0 (Serva). The resulting solution is filtered, aliquoted into 50-µL portions, and stored at –70°C. The original Servalyt solution is aliquoted into 0.5-mL portions and frozen at –70°C immediately after supply.

Table 2
Electrode Solutions for IEF

Components	Mixture	Final concentrations
Phosphoric acid	25 mL	7.27 % (w/v)
Urea	90 g	18.00 % (3 *M*)
Anode Solution	in 500 mL[a]	
Ethylenediamine	20 mL	5.00% (v/v)
Urea	216 g	54.00% (9 *M*)
Glycerol	20 g	5.00%
Cathode solution	in 400 mL[a]	

[a]Distilled water; the final volume is given.

Table 3
Equilibration Solution

Components	Mixture	Final concentrations
Tris buffer	250.00 mL[a]	125 m*M*
Glycerol	800.00 mL	40%
DTT	20.06 g	65 mM
SDS	60.00 g	3%
Equilibration solution	in 2000 mL[b]	

[a]Tris buffer: 30.275 g Tris in bidistilled water titrated with phosphoric acid; pH 6.8 at 20°C; final volume 250 mL.
[b]Bidistilled water; the final volume is given.

6. Electrode solutions: The composition is indicated in **Table 2**. The electrode solutions are prepared fresh before each IEF run and degassed for 5 min.
7. Equilibration solution: The composition is indicated in **Table 3**. Two liters of the final solution are filtered, aliquoted into 80-mL portions, and stored at –70°C.

2.2.2. SDS-PAGE

1. Gel solution: The composition is given in **Table 4**. The acrylamide solution is mixed with the buffer solution and the resulting solution degassed for 30 min in 1.5-L portions. SDS was added and dissolved by briefly and slowly stirring the solution, thus avoiding a new intake of air. The two 1.5-L portions are filtered and combined in this way in a large beaker (change the filter once). The gel solution is then aliquoted into 67.5-mL portions, frozen at –70°C, and stored at –20°C.
2. Ammonium persulfate solution: 3.2 g ammonium persulfate are dissolved in bidistilled water to give a final volume of 250 mL. The solution is then filtered, degassed, aliquoted into 10-mL portions, and stored at –20°C.
3. Underlayering solution: 20 g glycerol are dissolved in distilled water to give a final volume of 50 mL and stored at 4°C. Overlayering solution: 17.251 g Tris,

Table 4
Gel Solution for SDS-PAGE

Components	Stock solutions[a]	Mixture	Final concentrations
Acrylamide	498.000 g		15.000%
Bis	6.640 g		0.200%
	in 1660 mL[b]	1600.0 mL	
Tris	110.408 g		0.375 M[c]
Tris-HCl	45.436 g		
TEMED	0.960 g		0.030%
	in 1400 mL[d]	1400.0 mL	
SDS	—	3.2 g	0.100 %[e]
Gel solution		3000.0 mL[f]	

[a]The chemicals were dissolved in bidistilled water; the final volumes are given.

[b]The acrylamide solution was treated with Amberlite as indicated in **Table 1**, footnote[b]. To reduce the time, the solution is exposed to Amberlite after the 1 h of treatment, the main portion of the solution was decanted from the Amberlite at the bottom, and each part was filtered separately. In addition, if as much as 1660 mL have to be treated with Amberlite, we recommend dividing this volume into two halves.

[c]Molarity of Tris/Tris-HCl according to Laemmli *(15)*.

[d]pH of the buffer: 8.88–8.89 at 20°C (9.01–9.02 at 15°C); pH of the final gel solution: 8.80 at 20°C (8.96 at 15°C); pH given by Laemmli *(15)* for the gel solution: 8.8. The pH of the gel solution is critical for the migration behavior of the proteins: the lower the pH, the closer to the buffer front (or even within the buffer front) the proteins migrate *(15)*.

[e]According to Laemmli *(15)*.

[f]The increase in volume by adding 3.2 g SDS was ignored.

7.099 g Tris-HCl (Tris-buffer as indicated in **Table 4**), and 0.5 g SDS are dissolved in distilled water to give a final volume of 500 mL. The solution is aliquoted into 20-mL portions and stored at –20°C.

4. Agarose buffer: Tris/phosphoric acid buffer, pH 6.8, is prepared as indicated in **Table 3**. SDS (0.2 g; final concentration 0.1%) is dissolved in bidistilled water to give a final volume of 174 mL. To this solution, 25 mL Tris-buffer are added. The resulting solution is filtered, aliquoted into 9.95-mL portions, and stored at –20°C.

5. Electrode buffer: The composition is given in **Table 5**. The buffer is prepared fresh for each 2-DE run.

6. Bromophenol blue solution: 50 mg bromophenol blue are dissolved in 150 mL distilled water, filtered, aliquoted into 2-mL portions, and stored at –20°C.

7. Protein standards: 2-D SDS-PAGE standards from Bio-Rad (D-80939, München, Germany); phosphorylase B (rabbit muscle), β-galactosidase *(Escherichia coli)*, and myosin (rabbit muscle) from Sigma (D-82039, Deisenhoten, Germany); Rainbow Markers from Amersham Buchler (D-38001 Braunschweig, Germany); mol-wt markers, range 2512–16,949 from Pharmacia (D-79111, Freiburg, Germany); Gibco BRL 10-kDa Protein Ladder from Life Technologies GmbH (D-76344 Eggenstein, Germany).

Table 5
Electrode Buffer for SDS-PAGE

Components	Mixture	Final concentrations[a]
Tris	33.3 g	0.025 M
Glycine	158.4 g	0.192 M
SDS	11.0 g	0.100 M
Electrode buffer	in 11 L[b]	

[a]According to Laemmli *(15)*.
[b]Distilled water, the final volume is given; pH of the electrode buffer: 8.55 at 20°C (8.69 at 15°C); pH given by Laemmli *(15)*: 8.3.

Table 6
Solutions for Silver Staining[a]

Components of solutions		Amount of each component in 1 L[b] of each solution	Final concentrations
Fixation solution (1)	Ethanol	500.000 mL	50.00%
	Acetic acid	100.000 mL	10.00%
Incubation solution (2)	Ethanol	300.000 mL	30.00%
	Glutardialdehyde	20.000 mL	0.50%
	Sodium thiosulfate	2.000 g	0.20%
	Sodium acetate	41.015 g	0.50 M
Silver nitrate solution (3)	Silver nitrate	1.000 g	0.10%
	Formaldehyde	0.288 mL	0.01%
Wash solution (4)	Sodium carbonate	25.000 g	2.50%
Developer solution (5)[c]	Sodium carbonate	25.000 g	2.50%
	Formaldehyde	0.288 mL	0.01%
Stop solution (6)	EDTA	18.612 g	0.05 M
	Thimerosal	0.200 g	0.02%

[a]The table is based on data from the literature *(16,17)*.
[b]Distilled water; 1 L is the final volume.
[c]pH 11.3, adjusted with sodium hydrogen carbonate.

2.2.3. Silver Staining

Six different solutions are used for the silver-staining procedure, the composition of which is given in **Table 6**. The solutions are prepared fresh for the set of gels to be stained. In particular, the silver nitrate solution should be prepared shortly before use.

3. Method

3.1. Preparation of IEF Gels

1. Insert two glass tubes for 40-cm gels with nylon strings in start position (**Fig. 1**) into the tube stand (**Fig. 2**) (*see* **Note 11**).
2. Thaw one portion of each gel solution, and cap gel solution and ammonium persulfate solution. Incubate the gel solution for 30 min at 25–30°C in a waterbath. Immediately thereafter connect the vials containing the gel solution and the cap gel solution to a water-jet pump, and degassed for 5 min. The air bubbles usually stick to the wall and must be removed by knocking.
3. Mix the 25 µL ammonium persulfate solution (no vortexing!) into the gel solution, and pour the resulting solution into the gel solution groove of the tube stand (**Fig. 2**). Mix the cap gel solution with 10 µL ammonium persulfate immediately before use.
4. Use the procedure in the legend to **Fig. 1** for filling the glass tubes (*see* **Note 7**).
5. Leave the filled gel tubes for 20 min at room temperature to allow the gel solution to polymerize (**Fig. 1**).
6. Remove the gel tubes from the tube stand, place a drop of water at the opening of each tube end (without contacting the gel surface), and tightly close the ends with parafilm.
7. Leave the gels under these conditions for three nights (no less and no more) at room temperature (*see* **Note 13**).
8. Cast the 20 cm gels (*see* legend to **Fig. 1**) as described for the 40-cm gels. Use one portion of the gel solution to produce four or five 20-cm gels.

3.2. IEF Apparatus and Sample Application

1. Insert 4 tubes with 40-cm gels or 8 tubes with 20-cm gels into the upper chamber of the IEF apparatus with the cap gels directed downward.
2. Fill the lower (cathode) chamber with 400 mL ethylenediamine solution. Also fill the gel-free ends of the gel tubes with ethylenediamine solution.
3. Mount the two chambers one on the other with a cylinder in between, selected according to the length of the tubes.
4. Dry the surface of each gel with a fine strip of filter paper.
5. Mix one portion of the Sephadex suspension with 270 mg urea, 25 µL DTT solution, and 25 µL of the ampholyte mixture. (The Sephadex mixture is not reused.)
6. As follows from **step 2**, apply the protein sample to the anodic side of the gel. Layer sephadex, sample, protection solution, and phosphoric acid on the gel as shown in **Fig. 1** (*see* **Note 8**). This is performed with fine Pasteur pipets, except for the sample, which is applied precisely with a microliter syringe suitable for 10-µL volumes (type 701 RN + Chaney, Hamilton Bonaduz, Box 26, CH-7402 Bonadutz, Switzerland). Roughly, 8 µL and 100 µg of protein are applied to one gel.
7. Fill the upper (anode) chamber with 500 mL of phosphoric acid solution.

3.3. IEF Run

   The upper chamber of the IEF apparatus is connected to the anode, the lower chamber to the cathode of a power supply, and the electric current is regulated

Table 7
Regulation of Electric Current in 2-DE

IEF				SDS-PAGE[c]		
40-cm Gels		20-cm Gels				
Time schedule	V[a]	Time schedule	V[a]	Time schedule	mA	V[b]
1 h	100	1 h	100	15 min	65	150–160 (start)
1 h	300	1 h	200	6 h ± 5 min	85	210–230 (15 min after start)
23 h	1000	17.5 h	400			850–900 (final)
30 min	1500	1 h	650			
10 min	2000	30 min	1000			
		10 min	1500			
		5 min	2000			

[a]The mA and W regulators of the power supply were adjusted to the maximum.
[b]The volt regulator of the power supply was adjusted to the maximum.
[c]Two gels in parallel.

as indicated in **Table 7** (*see* **Note 12**). Within a series of experiments, always the same number of gels are run in parallel. Moreover, always the same electrophoresis chambers and the same power supply are used.

3.4. Equilibration and Storage of IEF Gels (see Note 14)

1. Thaw two portions of equilibration solutions, bring to room temperature, and distribute among eight Petri dishes (20 mL/dish). Fill several fine Pasteur pipets with glycerol (87%) that is slightly stained with Coomassie blue.
2. When the IEF run is finished, leave the tubes in the upper, empty chamber, and remove any solutions above the gel from both sides.
3. Remove the first tube from the chamber, and mark 40-cm gel tubes with a pencil 20 cm apart from the end (sample side) of the glass tube. More precisely: measure the half-length of the gel that usually shrinks somewhat during the run, and mark the tube according to this distance.
4. Fill glycerol into the free space below the cap gel, acting there as a protective layer (*see* **Note 9**). Using the nylon string (**Fig. 1**), extrude the gel directly into the incubation dish. When the gel appears on the tube end, quickly rinse the gel and tube end with distilled water before dipping the tube end into the incubation solution. When the end of the cap gel reaches the marking on the tube, cut the gel through with fine, sharp forceps and extrude the other half of the gel into a second dish (*see* **Note 10**).
5. Incubate the gels for 10 min by shaking at room temperature.
6. Suck the gel into a glass pipet with an appropriate inner diameter, and place it completely relaxed onto the gel groove (**Fig. 3A**). A minimum of solution cotransferred with the gel to the groove helps keep the gel relaxed.

7. Arrange the grooves carrying the gels of a run in a special box (**Fig. 3C**) and store at –70°C. The whole procedure, from finishing the IEF run to freezing the gels, must be performed as quickly as possible to restrict diffusion of the proteins within the gel. Furthermore, the incubation time of 10 min for each gel should not be exceeded, since protein becomes lost during this step. Consequently, perfect timing of the incubation step is necessary.

3.5. Preparation of Gels for SDS-PAGE (see Note 15)

1. Assemble two gel cells (**Fig. 4**) according to the instructions of the manufacturer using silicone grease to seal the cells instead of adhesive tape and silicone gaskets. Carefully clean the inner sides of the glass plates to remove any dust that may produce "point streaks" in the silver-stained protein patterns. Insert the two gel cells into the polymerization stand that has been placed on a horizontally adjusted plate.
2. Thaw two portions of gel solution and one portion of each ammonium persulfate solution and overlayering solution. Incubate the gel solutions for 30 min at 40°C in a water bath.
3. To each of the two portions of gel solution, mix 4.5 mL ammonium persulfate solution without vortexing (to avoid strong air contact with the gel solution). Immediately thereafter pour the gel solutions into the gel cells up to the edges of the glass plates using a special funnel (**Fig. 4**). Remove tiny air bubbles on the glass plates with a long, thin wire.
4. With the help of the syringe mounted on the bottom of the polymerization stand, slowly and steadily push 1.2 mL underlayering solution under the gel solution. Since in the meantime the gel solution has dropped a few millimeters, the gel solution still levels up with the edges of the glass plates.
5. Put a strip of parafilm onto the upper slit of each glass cell so that an air-free contact to the gel solution results. Allow the gel solutions to polymerize for 20–30 min.
6. Immediately (not later), turn upside-down and insert the gel cells into the gel stand, but not firmly fixed. Remove the underlayering solution, and rinse and cover the gel surface with overlayering solution. Leave the gels for 1 h at room temperature.
7. Completely remove the solution on the gel surface, turn around the cell (gel surface downward), put on wet filter papers, and store at 4°C overnight.

3.6. SDS-PAGE Apparatus and Transfer of the IEF gel

1. Prepare 9 L of fresh electrode buffer, fill the lower compartment of the electrophoresis apparatus, and precool to 15°C using a circulating cooling machine. Mix 2 L of the same buffer with 0.2 mL bromophenol blue solution, and use for the upper compartment.
2. Thaw and mix one portion of agarose buffer with 0.1 g agarose. Dissolve the agarose at 70°C and use at 40°C.
3. Bring the two gel cells prepared for the same apparatus into the right position.

4. Take two gel grooves, one carrying the "acid" half and one the "basic" half of a 40-cm IEF gel, from the –70°C refrigerator to transfer to the two gel cells (**Fig. 4**). When the gels have thawed, solution, if present in excess on the grooves, is sucked off, but left the gels wet to facilitate slipping of the gel between the glass plates without pushing. Hold the groove contact with the edge of the glass cell (**Fig. 3B**), and manipulate the gel with a special wire (**Fig. 3D**) onto the surface of the SDS-PAGE gel. This is done without stretching the gel. At the end of this step, the IEF gel should be in tight contact with the SDS-PAGE gel, i.e., inclusion of air or solution between the gels must be avoided. Suck off solution extruded to the ends of the IEF gel.
5. Overlay the IEF gels with agarose solution up to the edges of the glass cells (*see* **Note 18**). When agarose has gelatinized, insert the two cells into the electrophoresis apparatus.
6. Slowly fill the upper compartment of the apparatus with the electrode buffer.

3.7. SDS-PAGE Run

The electrophoresis apparatus is connected with the power supply using the upper buffer compartment as cathode and the lower compartment as anode. The electric current is regulated as indicated in **Table 7** (*see* **Note 17**). The lower buffer temperature is kept at 15°C (*see* **Note 16**).

The gel transfer, finished when starting electrophoresis, should be performed as quickly as possible to restrict diffusion of proteins. Furthermore, within a series of experiments, the same apparatus should always be used.

Electrophoresis was finished when the bromophenol blue line in the gels reaches the marking line of the glass plates (**Fig. 4**). The two gels are then immediately transferred, each into a glass trough containing 1 L fixation solution.

3.8. Silver Staining (see Note 19)

The gels are shaken for 2 h in the fixation solution and then left to stand in this solution overnight. Each gel is subsequently transferred into a large, transparent plastic trough (bottom 30 × 40 cm; Brukle-Labo-Plast, D-79540 Lörrach, Germany), which is carefully cleaned each time prior to use. The staining procedure is outlined in **Table 8** (*see* **Note 20**). During the whole procedure, the gels are left in the same trough under continuous shaking. After each step, the used solution is removed by means of a water-jet pump.

3.9. Gel Drying

1. Place the stained gel onto the perforated steel plate along with several sheets of Cellophane and filter paper as described in **Fig. 5**. Prior to this step, trim the edges of the gel below and lateral to the protein pattern to the size of the steel plate. Soak cellophane and filter paper in water (cellophane for several hours) before use. When layering the sheets on the plate, extrude excess water by hand.

Table 8
Silver Staining Procedure[a]

Gel treatment	Volume of solution per gel, identification no. of solution as indicated in **Table 6**	Length of treatment	Remarks
Fixation	1 L solution 1	2 h	Leave gel in solution 1 overnight
Incubation	1 L solution 2	2 h	
Wash	4 L distilled water	20 min	
Wash	4 L distilled water	20 min	
Silver reaction	1 L solution 3	30 min	
Wash	1 L distilled water	few s	Shake the gel just once by hand
Wash	1 L solution 4	1 min	Distribute arising gray clouds quickly
Pattern developing	1 L solution 5	3–5 min	Distribute arising gray clouds quickly, and watch the staining intensity
Development stop	1 L solution 6	20 min	Solution 6 intensifies the stain of the protein spots

[a]The table is based on data from the literature *(16,17)*.

2. Put the steel plate, when completely loaded, onto the drying plate installed in the incubator and further manage as described in **Fig. 5**. The gels dry by exposure to vacuum and 80–90°C for 1.5–2 h. The manometer drops during this period from about 150 mbar to almost zero.
3. The drying procedure is finished when the rubber tube is cool outside the incubator and dry inside the tube (use transparent polyethylene tubes).
4. Label the dried gels, and store the acid and basic halves of a gel together in a large envelope.

4. Notes
4.1. Quality Criteria for 2-DE Patterns

1. The quality of a 2-DE pattern can be judged on the basis of three criteria:
   a. Separation: In a 2-D pattern of high quality, the protein spots are sharp and well-concentrated; no streaks or background staining occurs.
   b. Resolution: In a high-quality 2-DE pattern of a complex protein sample (e.g., total tissue extract), several thousands of protein spots occur; they are well distributed over the whole gel area, they do not overlap and both are very weak, and tiny spots as well as very dark and large spots appear in the same pattern.

c. Reproducibility: High quality in this respect means:

   i. Several 2-D patterns resulting from the same sample match perfectly in all areas of the gel: In both directions, the absolute distance of corresponding spots from the start point of their gels is the same. Reproducibility can also be defined in a less stringent form as follows in (ii) and (iii).

   ii. The absolute position of corresponding spots of different patterns is, within a certain section of the 2-DE pattern, the same.

   iii. The relative position of corresponding spots of different patterns is the same, i.e., the relationship of the distances of one spot to several surrounding spots is for corresponding spots of different patterns in each pattern the same.

   In practice, definitions (ii) and (iii) are adequate, especially for large gels. We divide the large gels into a number of sections and compare sections instead of whole patterns. Computer programs for matching different 2-DE patterns use the higher reproducibility of the relative spot position (compared to the absolute position) to shift the corresponding spots of different patterns to the same absolute position. Thus far, we have considered the reproducibility of the position of the spots. However, patterns from different runs of the same sample should also show a high similarity with regard to the (relative) protein quantity of each spot.

2. 2-DE patterns that fulfill all the high-quality criteria mentioned cannot be realized. Differences in the nature of individual proteins (e.g., solubility, sensitivity to proteases) and protein samples *(see below)* exclude identical behavior of different proteins in the same 2-DE system. This becomes obvious when, within a narrow gel area, sharp and concentric protein spots occur side by side with streaking spots. We also made the observation that the quality of a 2-DE pattern depends to some extent on the origin of the protein sample. For example, protein extracts from embryonic mouse tissues generally result in protein patterns of higher quality than extracts from adult tissues; brain proteins gave better results than liver proteins. Moreover, some quality criteria are not compatible with each other; for example, revealing the weakest and finest spots of a protein pattern usually leads to streaks and overlapping among the dark and large spots.

3. **Figure 6** shows a protein pattern from human fibroblasts. The complexity of this protein sample was relatively low, and the variation in spot intensity did not reach extreme levels. Therefore, protein separation and resolution reached a quality level that can be considered as an optimum. In contrast, **Fig. 7** shows a pattern of high complexity (soluble fraction of mouse testis proteins), and highly abundant protein spots occur in certain areas. Consequently, more streaks and overlapping spots appear. On the other hand, the pattern reveals a maximum number of protein spots (we counted 10,345 spots; *see* **ref. 10**). Sections from this pattern in an enlarged format (**Fig. 8**) show that even in such a complex pattern, quality of spot separation is quite acceptable.

4. The high reproducibility that can be reached with the 2-DE technique described was demonstrated elsewhere *(10)*. Problems in obtaining reproducible protein patterns often result from the protein sample preparation rather than from the 2-DE
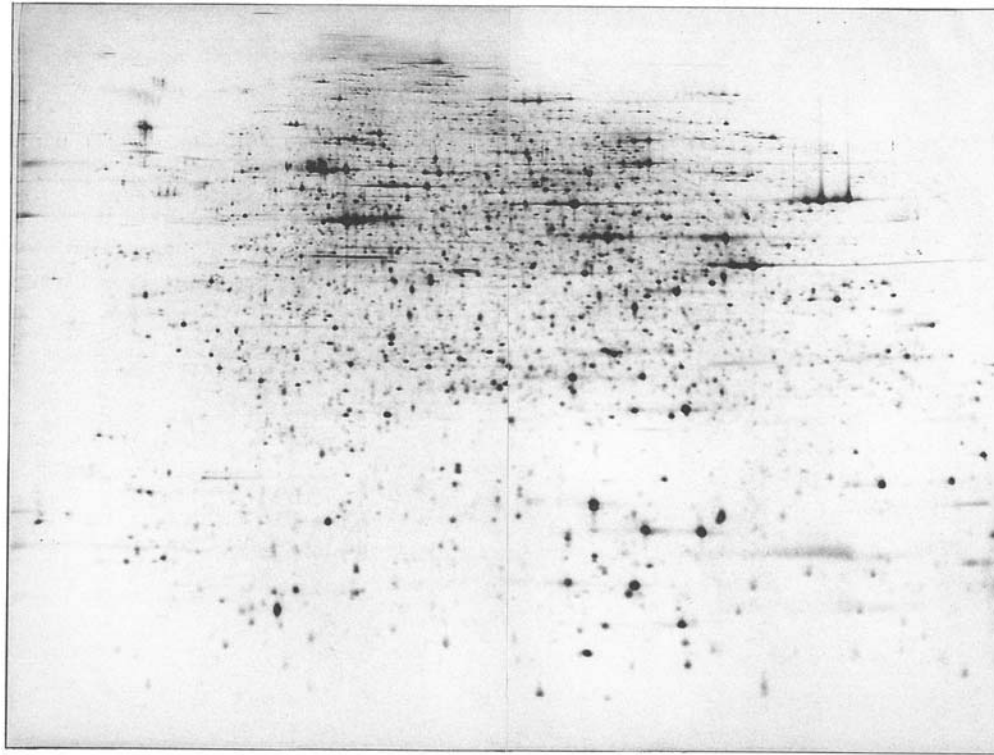
Fig. 6. Large-gel 2-DE pattern of total proteins from cultured human fibroblasts. 2-DE was performed as described in the text. The protein sample was prepared by mixing the cell pellet with buffer B/CHAPS (buffer factor 1.25; *see* Chapter 9) and homogenizing the suspension by sonication in a water bath. Urea, DTT, and ampholytes, pH 2.0–4.0, were added to the final concentrations of 9 *M*, 70 m*M*, and 2%, respectively (*see also* Chapter 9, Note 5). Sample load per gel was 75 μg protein/8 μL sample. The figure gives an example of a protein pattern that results when the protein sample is less complex and a clear background and well-separated spots are more desirable than a maximum of resolution. Patterns like these are advantageous for quantification and automated evaluation of protein spots.
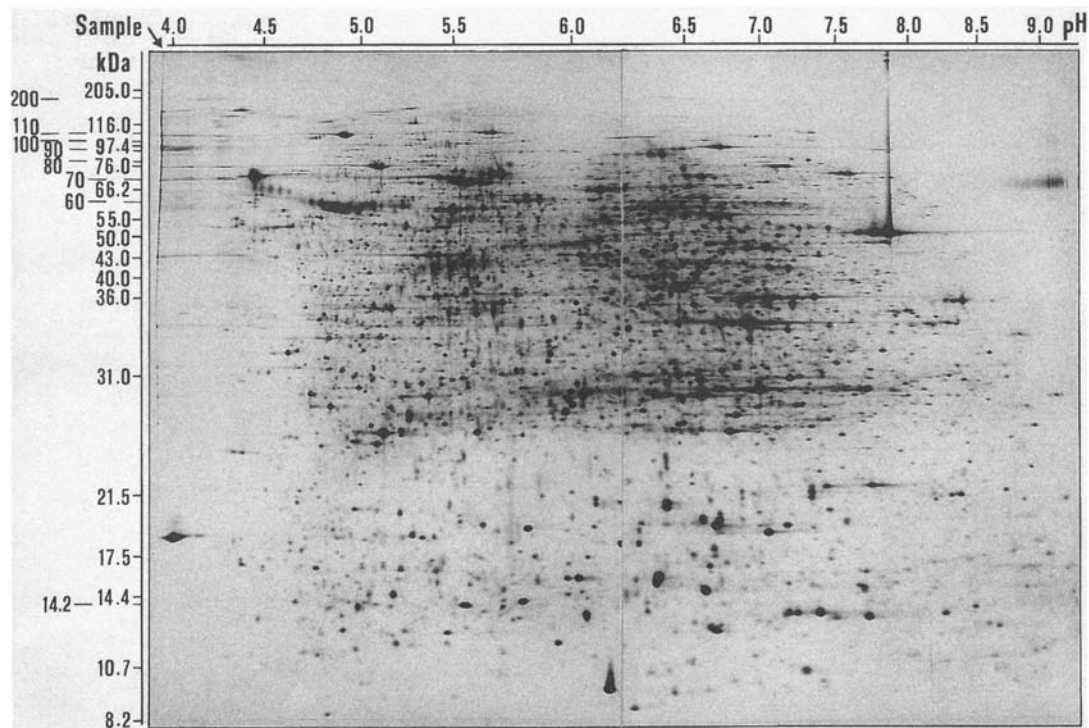
Fig. 7. Large-gel 2-DE pattern of soluble mouse testis proteins. 2-DE was performed as described in the text. Sample preparation followed the conception given in Chapter 9. Sample load per gel was 155 μg protein/10 μL sample. The pattern demonstrates a maximum of resolution. The protein spots of this pattern were counted by dotting each spot with an indelible pencil on a transparent plastic foil, which was put onto the dried gel (10); 10,345 spots were detected.
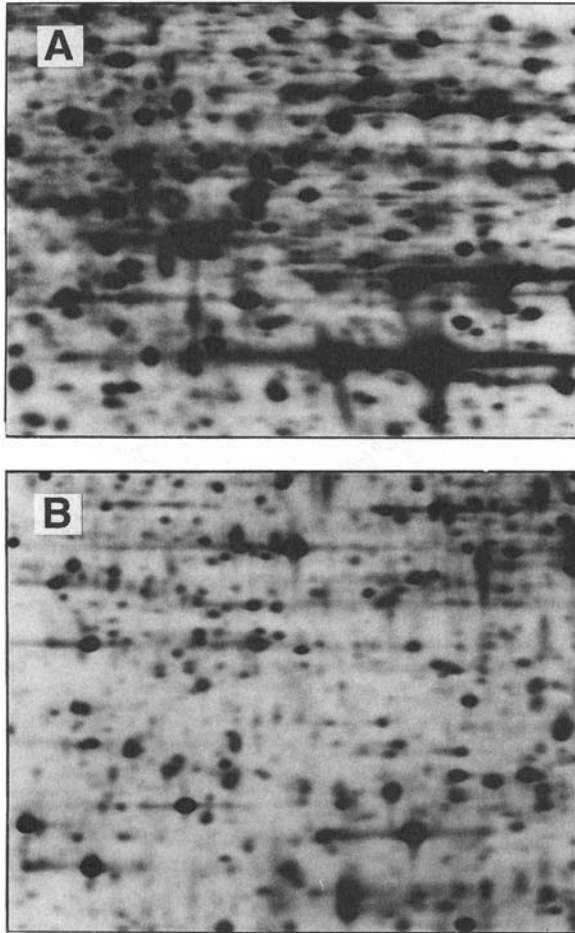
Fig. 8. Sections of the protein pattern shown in **Fig. 7**. **(A)** Section of the right side of the pattern, the most crowded area. **(B)** Section of a rather light area of the left side of the pattern.

procedure itself. Furthermore, proteins have an amphoteric character and, there-fore, may modify the pH gradient in the IEF gel, or in a distinct region of the gel in which a highly abundant protein dominates the pH. This problem may also occur in immobilized pH gradients. Consequences of this problem for the repro-ducibility may become obvious when protein patterns of quite different sources (e.g., serum protein versus brain proteins) are compared. Lack of reproducibility of single spots may occur in the basic region of the 2-DE gel rather than in the acid region. This is because under our IEF conditions, the basic part of the pH gradient does not reach its equilibrium.

4.2. Buffers and Solutions

To reach high reproducibility of the 2-DE patterns, the gel solutions for IEF and SDS-PAGE were produced in large batches, almost complete in their composition, and stored in ready-made portions at –70°C. For a set of experiments, gels were prepared from the same batch of solutions. Disadvantageous effects were not observed when the gel solutions were stored frozen for more than 2 yr. Source and quality of the chemicals used should always be the same, once found to be satisfactory. This is noteworthy particularly with regard to ampholytes and SDS.

4.3. Isoelectric Focusing (IEF)

6. Thin IEF gels (0.9 mm) and thin SDS gels (0.75 mm) were found to give sharper spots and therefore better resolution than thicker gels (1.5–5 mm). If, however, protein spots have to be extracted from the gels for amino acid sequencing and other analytical purposes, 1.5-mm gels in combination with higher sample load (50–60 μL containing 0.6–0.8 mg protein) still resulted in protein patterns comparable with that of thin gels.

7. Layering several solutions in 0.9-mm tube (Sephadex, protein sample, protection layer, phosphoric acid) on the IEF gel is difficult and requires some experience. Very thin pipets should be used (except for Sephadex solution), and the surface of the lower layer should be touched with the tip of the pipet before extruding the solution.

8. The function of the Sephadex layer is to protect (to some extent) the gel surface from shrinking and becoming plugged by protein precipitates, which may occur when the proteins become highly concentrated above the gel shortly after the start of IEF.

9. The function of the cap gel is to prevent the end of the separation gel from shrinking during the run, since otherwise the electrode solution might ascend between the gel and tube wall, thus destroying the pH gradient *(9,13,14)*.

10. Extruding the long and thin IEF gels out of the glass tubes using the nylon string as described usually works without any problems. In this respect, the use of piperazine diacrylamide for IEF gels instead of *bis*-acrylamide is important, since the stability of the gel is increased in this way. Occasionally, however, the gel breaks in the basic region of the gel, near the cap gel. In this region, IEF gels become slightly thinner during the IEF run, and this process is influenced by the protein sample, i.e., by the concentration of proteins and ampholytes in this region. For example, protein extracts from cell membranes or from cell nuclei, which contain more basic proteins, affect in this region the gel matrix more than proteins from the cytoplasm of the cells. If breaking of the gel becomes a problem, the amount of the protein sample per gel should be reduced to some extent. Furthermore, the surface properties of the inner wall of the glass tube have an effect on the adhesion of the gel to the glass and possibly on the polymerization of acrylamide. We observed when extruding the gel out of the tubes that the gels slipped out more easily when the tubes had already been used several times. This

was probably owing to changes on the glass surface that occurred with use. It seemed to us that the procedure of cleaning the tubes also had an effect on the glass surface. Therefore, we recommend cleaning the tubes in the way described below.

11. Cleaning the glass tubes: The used glass tubes are rinsed by pressing distilled water through the capillary canal. The tubes are then kept for 30 min at 60–80°C in a glass cylinder containing a 6% solution of Deconex 12 PA (Borer Chemie, CH-4528 Zuchwil, Switzerland). During this period, the tubes are moved once up and down using a metal stand for the tubes in the glass cylinder. Then the tubes are rinsed with water as described and kept for 30 min at 95°C in a 0.1 N HCl solution. The tubes are rinsed again, dried by air pressure, and stored under dust-free conditions. The heating steps are performed in an incubator using pre-heated solutions.

12. Owing to the high ionic strength of ethylenediamine in the lower electrode solution, very basic proteins (e.g., histones) accumulate at the end of the gel (before the cap gel) rather than moving out of the gel. If this group of proteins is of particular interest (*see* Chapter 9, Fig. 2, the pellet suspension), the running time for IEF should be reduced by 2 h at the 1000-V level to obtain a better spreading of these protein spots.

13. IEF gels, when prepared and polymerized in glass tubes, were kept at room temperature for three nights. This improves the reproducibility of IEF probably because polymerization of the soft gels reaches the final stage. Furthermore, we occasionally observed the appearance of diagonal streaks at some protein spots of the 2-DE pattern, particularly at the prominent spots. This might be owing to interactions of these proteins with solubilized components of the gel. This phenomenon occurred to a lower extent, or not at all, if the gels were kept at room temperature for three nights.

4.4. Equilibration of IEF Gels

14. The purpose of incubating the IEF gels in the equilibration solution is to expose the proteins to a high concentration of SDS before separating them by SDS-PAGE. However, at this step a portion of proteins becomes lost by diffusion into the solution. The disadvantage is that diffusion may affect the proteins to a different degree: Small proteins and proteins that do not focus perfectly (and therefore still preserve their solubility) may become preferentially lost. We, therefore, reduced the incubation time to 10 min. Satisfactory results were also obtained when the gels were just rinsed for a few seconds in the equilibration solution prior to use or freezing. However, in this case background staining and streaking in the SDS gel may be enhanced. If this is observed, the sample load should be slightly reduced.

4.5. SDS-PAGE

15. A large-gel format was used to increase the resolution of protein mixtures. The increase of resolution is achieved in this way by increasing the distance between the protein spots. The free space gained in this way can then be used to reveal additional spots by increasing the sample load or staining period. Compared to

this, separation of completely superimposed spots by increasing the gel size contributes just slightly to the increase in spot number. This is owing to the fact that completely superimposed spots rarely occur even in normal-sized (20 × 30 cm) SDS gels. Increasing the gel size increases also the diffusion of protein spots because of the increased running time, and this reduces the free space gained. The limited complexity of the ampholyte mixtures also limits the increase in resolution caused by increasing the gel size. Therefore, increasing the gel size more and more does not result in a linear increase of resolution.

16. The temperature during the SDS-PAGE run should be 15°C and not much lower. Otherwise a temperature gradient may arise across the gel: the temperature in the center of the gel is higher than at the two surfaces of the gel. Consequently, the protein molecules do not migrate in the same front, and this affects the shape of the spots.

17. For blotting and extracting proteins thicker gels, i.e., 1.5 mm, IEF gels and SDS gels were used. In this case, the SDS gels were run at 120 mA and after 15 min at 150 mA.

18. Embedding the IEF-gel in agarose can be omitted. This may result in sharper spots in the lower part of the SDS gel.

4.6. Silver Staining

19. The quality of the stained protein pattern (background, staining intensity) depends on the quality of the chemicals used (purity, age), and this is particularly true for ethanol, formaldehyde, and glutaraldehyde. Furthermore, the quality of the water is crucial for the stained pattern; the conductivity of the water should be below 0.1 µS/cm.

20. Thimerosal in the stop solution acts, for unknown reasons, on the stained spots as an intensifier and gives the spots a more homogeneous gray-violet color. However, the red centers in the highly concentrated protein spots and some differences in the color of the spots (problems relevant to densitometric measurements) were not avoided completely. Inhibiting the staining process by developing at 4°C, using less formaldehyde or increasing the *bis* concentration of the separation gel did not solve this problem. In this respect, the use of Duracryl (Millipore, Bedford, MA) instead of acrylamide/*bis* for the separation gel was found to be advantageous to a certain extent, even when the staining method described here was used.

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19

Advantages of Immobilized pH Gradients

Jenny Fichmann

1. Advantages of Immobilized pH Gradients

Dried gel strips containing immobilized pH gradients (IPG) were commercially introduced in 1991 (Pharmacia Biotech, Immobiline® DryStrip gels). Their adoption for the first dimension of 2-D electrophoresis has produced significant improvements over the classical O'Farrell carrier ampholyte-based 2-D electrophoresis separations.

In the original 2-D electrophoresis method, the required pH gradient is established by the migration of individual species of carrier ampholytes to their respective isoelectric (p*I*) points. Batch-to-batch variations of the complex carrier ampholyte mixtures result in variations in the shape of the pH gradient. The use of commercially prepared immobilized pH gradient gels, IPG DryStrip, introduced by Bjellqvist et al. *(1)*, and Gorg et al. *(2)* eliminates the batch-to-batch variations. The pH gradient is immobilized by covalently incorporating Immobiline® acrylamido buffers into the acrylamide matrix during polymerization. Since Immobiline consists of discrete, relatively simple molecules, they can be manufactured very reproducibly pure, eliminating batch effect and reproducibility as demonstrated by interlaboratory comparisons *(3)*. Further, p*I* resolution to 0.01 pH unit can be achieved *(1)*. The acrylamide matrix with the Immobiline, acrylamido buffers is cast onto a GelBond backing sheet, polymerized, washed, and dried. The backing gives the strips size stability and simplifies handling. The dried strips can be rehydrated in various buffers and additives that would inhibit polymerization if included at the time of casting.

The shape of the pH gradient in carrier ampholyte-based 2-D is influenced by the different protein and salt concentrations. Further, over time, the gradient

drifts, and part of the acidic and almost the entire basic end of the gradient is lost. Thus, it has never been possible to display the entire range of proteins from a cell or a tissue. Separation in the basic range could be improved with nonequilibrium pH gradient electrophoresis (NEPHGE) gels. Since this is not an equilibrium technique, reproducibility is not easily achieved and true p*I* values are not reached. IPG strips dramatically improve the reliable basic protein separations (p*I* > 10) *(4)*. In addition, for micropreparative and preparative runs, protein loading capacity is greatly improved and does not affect the shape of the gradient.

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20

Casting Immobilized pH Gradients (IPGs)

Elisabetta Gianazza

1. Introduction

One of the main requirements for a 2-D protocol is reproducibility of spot position, and, indeed, the technique of isoelectric focusing on immobilized pH gradients (IPGs) is ideally suited to provide highly reproducible 1-d separations. IPGs are obtained through the copolymerization of acidic and basic acrylamido derivatives of different pKs within a polyacrylamide matrix *(1,2)* (**Fig. 1**). The pH gradient may be devised by computer modeling either with a linear or with an exponential course. IPGs are cast from two limiting solutions containing the buffering chemicals at concentrations adjusted to give the required pH course upon linear mixing. For consistent results, gradient pouring and polymerization are carried out under controlled conditions. The covalent nature of the chemical bonds formed during the polymerization step results in a permanent stability of the pH gradient within the matrix. Conflicting requirements during the focusing procedure prevent any effective use of IPGs into capillary tubes *(3)*: The need to buffer with carrier ampholytes (CAs) the pH extremes caused by the migration of the polymerization catalysts is contrasted by the adverse effects of the electroendosmotic flow brought about by the addition of CAs to the gel phase. The demand for the IPG gels to be backed by a binding support—they are usually cast on GelBond™ foils—results in dimensional stability between 1-d and 2-d as a further assistance to reproducibility.

Toward the aim of reproducibility, batch production and quality control as allowed by an industrial process give the commercially available IPG strips (Immobiline DryStrip™ from Pharmacia, Uppsala, Sweden) obvious advantages over homemade slabs. Moreover, the pH course of the commercial product is being carefully characterized in chemicophysical terms, by assessing the dissociation constants of the acrylamido buffers under the experimental conditions relevant to
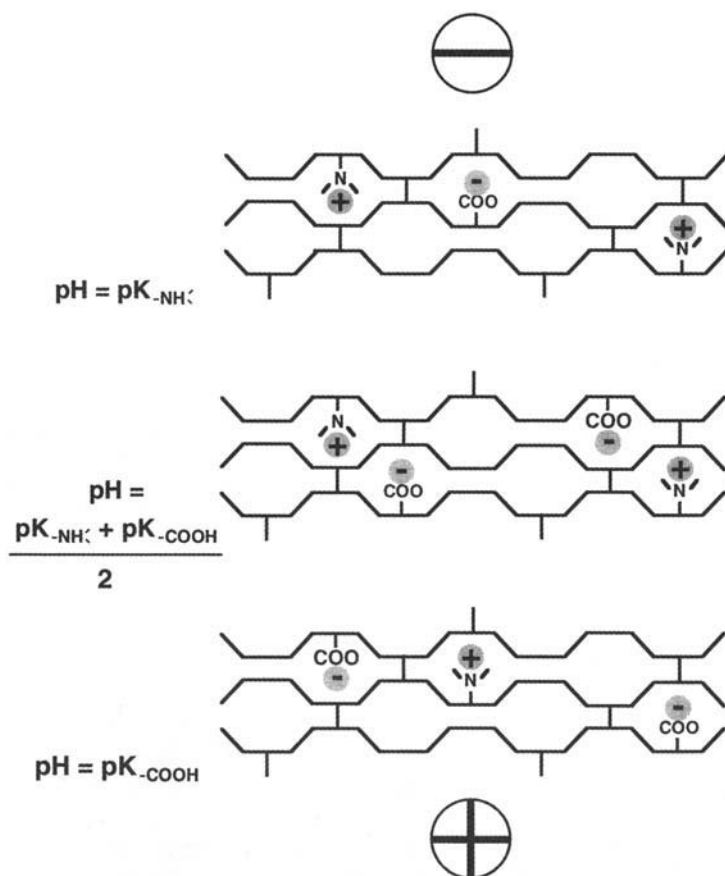
Fig. 1. Structure of the IPG matrix. Different regions of an IPG made up with a basic and an acidic Immobiline are schematically drawn from cathode to anode. In each region, pH depends from the concentration ratio between the basic and the acidic dissociating groups. From the Henderson-Hasselbalch equation, where the concentration of the basic monomer is twice the concentration of the acidic monomer, pH equals the pK of the base, where the concentration of the basic monomer is one-half the concentration of the acidic monomer pH equals pK of the acid, and where the two concentrations are alike, pH equals the mean between the two pKs.

the 1-d run of an IPG-DALT *(4)*. The aim of this effort is to connect focusing position reliably with p*I (5)*; any discrepancy between computed and experimental values for known proteins would then hint at the occurrence of posttranslational modifications *(6)*.

From the above it seems this chapter could shrink to the statement: Use the ready-made IPG strips according to the manufacturer's instructions. However,

there are reasons, and not only of economical order, for laboratories to cast their own slabs. The main one is the variability of the analytical needs. 2-D protocols usually aim at the resolution of all peptide components of a complex mixture. Although the proteins in cellular extracts and biological fluids have mostly mildly acidic p*I* values, this is not true of all samples, and the optimal pH course should be devised accordingly. Moreover, after specific qualitative or quantitative variations have been detected for some spots as the result of a given experimental treatment, it is usually worth investigating the area of interest under conditions of maximal resolution. This is especially true if the spots have to be identified or characterized, e.g., by mass spectrometry (MS) techniques after a blotting step *(7)*. IPGs allow the tailoring of wide, narrow, or ultranarrow pH gradients, whereas migration on continuous or gradient PAA slabs of different %T in 2-d may further improve resolution of the spots of interest.

In order to optimize reproducibility and to increase throughput, laboratories handling a very large number of gels may consider a medium-scale production with gradient pouring through mechanical devices (computer-driven burettes) *(8,9)*. In any event, even at the typical laboratory level, the procedure of IPG casting is reliable and allows a remarkable reproducibility of results *(10)*. The following will try to convince the readers that it is also an easy one.

2. Materials

2.1. Equipment

1. Polymerization cassette:
   a. A molding plate with a 0.5-mm-thick permanent frame. Available from Pharmacia in either 12.5 × 25 cm, w × h (cat. no. 18-1013-74), or 25 × 12.5 cm, w × h, size (cat. no. 80-1106-89).
   b. Gel-supporting plate.
   c. Gel-binding foil, e.g., GelBond PAG™ (Pharmacia).
   d. Clamps.
2. Gradient-mixing device:
   a. A two-vessel chamber (e.g., gradient maker from Pharmacia, cat. no. 18-1013-72).
   b. Magnetic bars.
   c. Outlet to the mold, either a one-way silicone tubing (od 3 mm, id 1.5 mm, ca. 12 cm long, equipped with a 2-cm teflon tip) or multiple inlets cast by adapting the tips, ca. 6 cm long, of butterfly needles gage 21 to a T-connector, available from Cole-Parmer (cat. no. K6365-70).
   d. A stirrer providing constant and even operation at medium to low rpm rating with minimal overheating.
   e. Screw-jack rising table.
3. Electrophoresis apparatus:
   a. Horizontal electrophoretic chamber with movable electrodes.

Table 1
pK Values of Immobilines™

Chemicals	Water solution, 25°C[a]	PAA gel, 10°C[a]	8 M urea in PAA gel, 10°C[b]
Immobiline pK 3.6™	3.58	3.57	4.47
Immobiline pK 4.6™	4.61	4.51	5.31
Immobiline pK 6.2™	6.23	6.21	6.71
Immobiline pK 7.0™	6.96	7.06	7.53
Immobiline pK 8.5™	8.52	8.50	8.87
Immobiline pK 9.3™	9.27	9.59	9.94

[a]From LKB Application Note 324.
[b]From **ref. 42**.

    b. Power supply able to deliver >3000 V. We recommend using a power supply with an amperometer sensitive to <1-mA currents.
    c. Thermostating unit.
4. Forced-ventilation oven at 50°C.
5. Shaking platform.
6. Fan.

2.2. Reagents

1. Acrylamide buffers: 0.2-*M* solutions (*see* **Table 1**), prepared either in water with 5 ppm of hydroquinone methylether as polymerization inhibitor for the acidic monomers or in *n*-propanol for the basic monomers. The chemicals are available from Pharmacia (as Immobiline™) or from Fluka (Buchs, Switzerland). Storage at 4°C; the expiration date is given by the manufacturer. The products are defined as irritant, whereas *n*-propanol is classified as highly flammable.
2. Acrylamide monomers stock solution: 30% T, 4% C *(11)*. Dissolve in water 1.2 g *bis*-acrylamide and 28.8 g acrylamide/100 mL final volume. Filter the stock through 0.8-mm membranes and store at 4°C for about 2 mo. The chemicals should be of the highest purity. Acrylamide has been recognized as a neurotoxin.
3. 40% w/v Ammonium persulfate solution: Dissolve 200 mg of ammonium persulfate in 440 μL of water. Store up to a week at 4°C.
4. TEMED: Store at 4°C for several months.
5. 87% v/v Glycerol.
6. 1 *M* Acetic acid: Dilute 57.5 mL of glacial acetic acid to 1 L.
7. Dimethyldichlorosilane solution: 2% v/v solution of dimethyldichlorosilane in 1,1,1,-trichloroethane. Both chemicals are available from Merck (Darmstadt, Germany), or a ready-made solution is marketed by Pharmacia as RepelSilane™.
8. Glycerol washing solution: 1% v/v glycerol.

3. Method

3.1. Selecting a Formulation

The formulations for narrow to wide-range IPGs have been reported in a series of papers *(12–15)*, and the recipes for casting the gradients collected *(2)*. Most of the formulations use the acrylamido buffers marketed as Immobiline™ from Pharmacia, since the pK distribution in their set is even and broad enough for all applications in the 4.0–10.0 pH region. For specific purposes—increased hydrophilicity, narrow ranges, pH extremes—other chemicals are available as acrylamido derivatives *(16)* or have been custom-synthesized *(17,18)*. These chemicals are available from Fluka.

Computer programs for pH gradient modeling, with routines optimizing the concentrations of the acrylamido buffers required in the two limiting solutions in order to give the expected pH course, have been described in the literature *(19–21)*. One of these programs is available from Pharmacia (Doctor pH™). For most practical applications, a wide pH gradient is required for 2-D mapping in order to resolve all components of a complex biological sample. A statistical examination of the p*I* distribution across all characterized proteins as well as the typical maps of most biological specimens show a prevalence of acidic to mildly alkaline values over basic proteins. A nonlinear pH course is then expected to give improved resolution of most proteins in a complex sample. An exponential gradient whose slope increases from pH 6.0 to 7.0 and to 8.0 optimally resolves most samples run in IPGs: one such recipe *(14)* is given in **Table 2** (I), whereas the gradient is plotted in **Fig. 2**.

The narrow ranges in **Table 2** (II–IV) have been interpolated from the sigmoidal gradient above, and provide a better resolution of the acidic, neutral, and basic protein components (**Fig. 3**) while maintaining the same slope ratios among the different pH regions. Alternatively, resolution may be improved by the parallel use of two linear, partly overlapping gradients, namely 4–7 and 6–10 *(12)* (**Table 3**).

3.2. Preparing the Working Solutions

As an equilibrium technique, IPG technology freezes within the structure of the amphoteric matrix the actual composition of the acrylamido buffer mix. Since there is no adjustment once the gradients are cast, all steps in liquid handling require the careful use of properly calibrated measuring devices.

1. On two calibrated test tubes or 10-mL cylinders, mark the pH and the volume of the solutions to be prepared with a felt-tip (*see* **Note 1**).
2. Add the acrylamide buffers. For reproducible measurements, all chemicals should be at room temperature (*see* **Note 2**).
3. After adding all the acrylamido buffers, fill the two solutions to one-half their final volume with water, and check their pH with a microelectrode. Refer to

Table 2
IPG 4-10 with a Nonlinear Course[a]

	Recipes for 5.1- + 5.1-mL Gradients							
	I: Whole range		II: Acidic region		III: Neutral region		IV: Basic region	
Chemicals	A[b] μL	B[c] μL	A μL	B μL	A μL	B μL	A μL	B μL
pK 0.8	135	16	135	62	103	45	86	14
pK 4.6	130	—	130	52	98	33	78	—
pK 6.2	153	46	153	89	126	73	110	46
pK 7.0	—	61	—	37	15	46	24	61
pK 8.5	—	23	—	14	6	17	9	23
pK 9.3	—	43	–	26	11	32	17	43
1 M Acetic acid	—	14	—	8.4	3.5	10.5	4.6	14
T30 C4	700	700	700	700	700	700	700	700
87% Glycerol	1000	—	1000	—	1000	—	1000	—
TEMED	3.1	2.7	3.1	2.9	3	2.8	2.9	2.7
40% APS[d]	4.75	4.75	4.75	4.75	4.75	4.75	4.75	4.75
pH at 25°C[e]	4.13	9.61	4.13	6.27	4.67	7.07	5.25	9.61

[a]From (14); pH gradient course in **Fig. 2**.
[b]Acidic solution.
[c]Basic solution.
[d]To be added to the solutions after transferring to the gradient mixer and starting the stirrer.
[e]pH of the limiting solutions.

expected pH readings at 25°C in the liquid phase, not the values computed for 10°C in the gel phase (*see* **Tables 2** and **3**).

4. Add acetic acid to the basic solution to bring the pH to between 7.0 and 7.5 in order to prevent hydrolysis of the amide bonds during the polymerization step.

5. Add glycerol to the acidic solution to a final concentration of 15–20% v/v. Use a wide-mouth or a clipped pipet tip for dispensing the 87% glycerol stock.

6. The solutions are filled to their final volume with water and carefully mixed. Acrylamide is typically used at the final concentration of 4% T, 4% C. The concentration of TEMED is higher in the acidic than in the basic solution to counteract the effect of amine protonation. Ammonium persulfate is **not** added at this stage (*see* **Note 3**).

3.3. Assembling the Polymerization Cassette

To obtain a 16-cm high IPG slab that fits the 2-d SDS-PAGE gel (e.g., Protean™ from Bio-Rad [Hercules, CA]), the 12.5 × 25 cm w × h cassette is used (*see* **Note 4**).
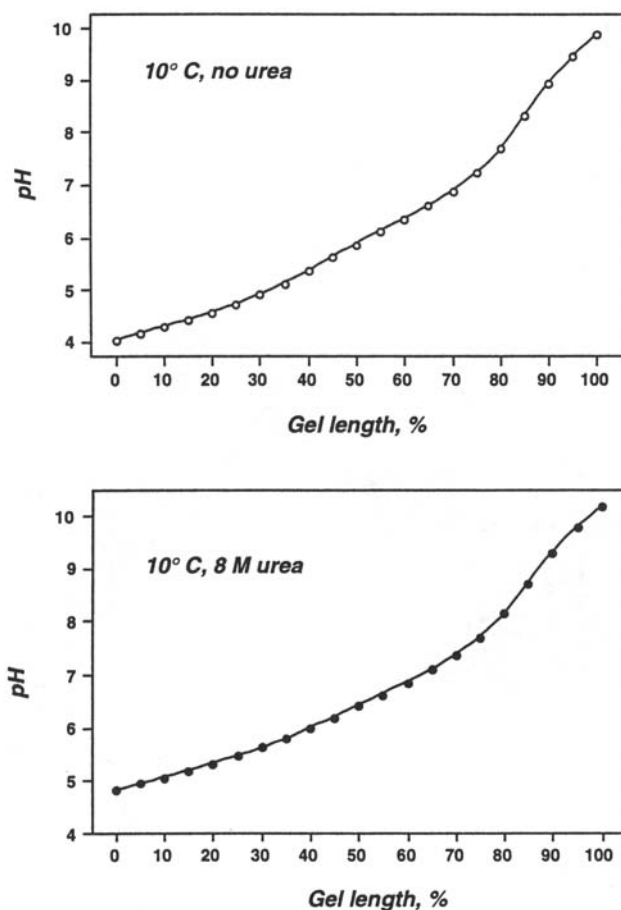
Fig. 2. Course of the gradient for the 4–10 nonlinear IPGs. The top panel shows the pH course computed for the gradient in **Table 2** *(14)* after grafting into a PAA matrix (pK values from **Table 1**, 2nd column); the bottom panel corresponds to the pH values corrected for the presence of 8 *M* urea (pK values from **Table 1**, 3rd column).

1. Mark a GelBond PAG™ foil for polarity using a felt-tip marker (anode at the bottom, cathode at ⅔ height); additional annotations (pH gradient, date, even short reminders) may help further identification. The felt-tip marking does not interfere with electrophoretic procedures (*see* **Note 5**).
2. Wet the gel-supporting plate with distilled water.
3. Lay the GelBond PAG™ foil—hydrophobic side down, hydrophilic side up, still covered with the protective paper foil—on a gel-supporting plate. Adhere the foil to the plate by rolling it onto the glass. Care is taken to align the foil flush with the plate.
4. Discard the protecting sheet. Blot any water on the hydrophilic side of the foil with a paper tissue.
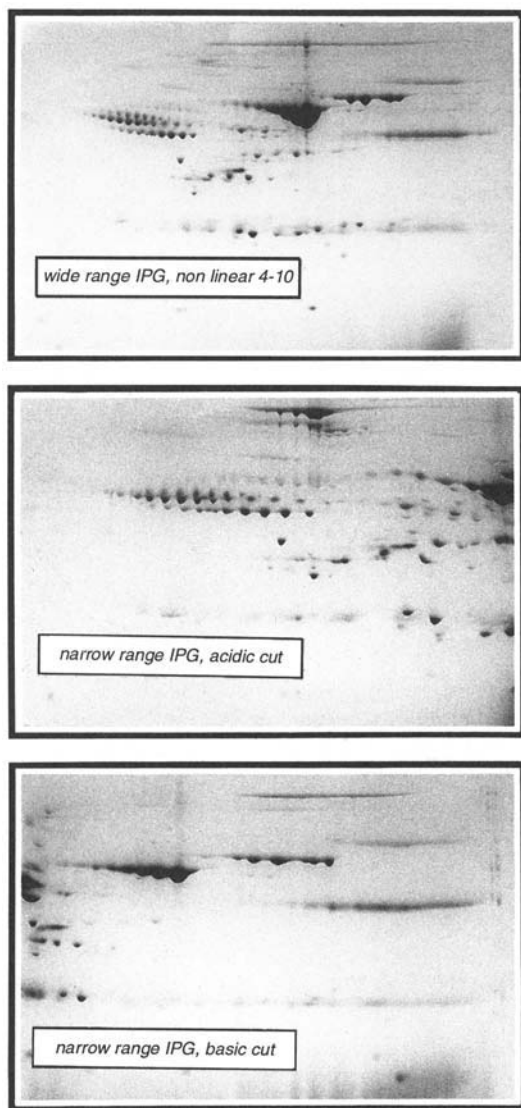
Fig. 3. Examples of IPG-DALT separations. Rat serum was run in 1-d on the 4–10 nonlinear IPG in **Table 2** (I) (top panel) and on its narrow cuts, also in **Table 2** (acidic region [II] in middle panel, basic region [IV] in bottom panel). The 2-d run was on a 7.5–17.5% T PAA gradient with the discontinuous buffer system of Laemmli *(24)*; the samples from narrow-range IPGs were overrun in order to improve resolution along the *y*-axis (the above runs from a work in preparation with Haynes, P., Miller, I., Aebersold, R., Gemeiner, M., Eberini, I., Lovati, M. R., Manzoni, C., Vignati, M., Gianazza, E. Proteins of rat serum: I. Establishing a reference two-dimensional electrophoresis map by immunodetection and microbore high-performance liquid chromatography. *Electrophoresis*, in press.).

Table 3
IPG 4-7 and 6-10 with a Linear Course[a]

| | Recipes for 5.1- + 5.1-mL Gradients | | | |
| | pH 4.0–7.0 | | pH 6.0–10.0 | |
Chemicals	A[b] μL	B[c] μL	A μL	B μL
pK 3.6	197	103	329	35
pK 4.6	37	251	—	—
pK 6.2	153	51	96	117
pK 7.0	—	91	85	127
pK 8.5	—	—	91	83
pK 9.3	—	298	99	114
1 *M* Acetic acid	—	—	—	40
T30 C4	700	700	700	700
87% Glycerol	1000	—	1000	—
TEMED	3.1	2.7	2.8	2.7
40% APS[d]	4.75	4.75	4.75	4.75
pH at 25°C[e]	4.02	6.93	5.99	9.72

[a]From **ref. *12***.
[b]Acidic solution.
[c]Basic solution.
[d]To be added to the solutions after transferring to the gradient mixer and starting the stirrer.
[e]pH of the limiting solutions.

5. Swab the inside of the molding plate with a paper towel moist with a dimethyldichlorosilane solution. It is recommended to wear gloves and to perform this treatment in a fume hood.
6. After the solvent has dried out, rinse the surface of the glass with distilled water, and dry with a paper towel. Inspect the rubber gasket for any break or any adherent material (dried-out acrylamide, paper cuts, and so forth).
7. Invert the molding plate on the GelBond-glass assembly, and carefully align it. Check the marked polarity!
8. Fasten the cassette with clamps of adequate thickness and strength. If two strips from the 1-d IEF are to be aligned tail-to-head for the 2-d SDS-PAGE run on the same gel, the procedure detailed above applies to the 25 × 12.5-cm w × h mold. Pouring a gradient of the stated volume (**Table 2**) will result in an 8-cm-high gel.

3.4. Pouring the Gradient

1. Add the magnetic bar to each chamber of the gradient mixer. Set both connections between the two vessels as well as to the outlet tubing in the closed position. Arrange the mixer on a stirrer, about 5 cm above the mouth of the polymerization cassette.

2. Stick the outlet(s) (*see* **Subheading 2.1., step 2**) in the cassette taking extreme care to not miss the opening to reach instead the gap between GelBond and supporting glass plate.

3. Transfer the acidic, heavy solution into the mixing chamber of the gradient mixer (i.e., the vessel next to the outlet).

4. Briefly open the connection to the reservoir in order to purge air bubbles from the solution; return the solution that flowed into the reservoir during the purging operation to the mixing chamber with a Pasteur pipet.

5. Add the basic light solution to the reservoir.

6. Start the mixing on the stirrer at high speed. Slowly move the gradient mixer around the active area of the mixer in order to find a position in which the magnetic bars in both chambers turn evenly and synchronously. Reduce the stirring speed.

7. Add ammonium persulfate solution in the stated amounts (*see* **Table 2**) to the solutions in both chambers.

8. Open the outlet to the polymerization chamber; the liquid should easily flow by gravity: in case it does not, a light pressure (the palm of your hand over the mouth of the vessel) might solve the problem.

9. When the heavy solution is about to enter the polymerization cassette, open the connection between the two chambers of the mixer.

10. The in-flowing light solution should mix promptly with its heavy counterpart; if not (streams of liquid of different density are distinctly seen in the mixing chamber), the stirring speed should be increased accordingly (*see* **Note 6**).

11. When all solution has flown by gravity, force the few drops remaining in the outlet tubing and in the mixing chamber to the cassette by applying pressure *(see above)* first on the reservoir and then on the mixing chamber, while raising the gradient mixer.

12. Inspect the liquid within the cassette: no air bubbles should be present. It is possible to remove them, with **great caution**, with the help of a thin hook cut from GelBond foil.

13. Leave the gradient to stabilize for 5–10 min at room temperature before moving the cassette into the oven to start the polymerization step.

14. After use, promptly fill the gradient mixer with distilled water and flow the water through the outlet tubing; repeat this procedure twice.

15. Set aside the stirring bars; disassemble the outlet tubing and dry by suction with a pump; and blow the liquid still remaining inside the gradient mixer with compressed air, before drying the outside with a paper tissue.

16. Let the equipment dry to completion with all connections in the open position. Rinsing and drying the gradient mixer, and pouring a new gradient takes ~10 min, so no delay is actually required when processing a number of IPGs one after another.

3.5. Polymerization Conditions

1. Let the IPGs polymerize for 1 h at 50°C (*see* **Note 7**).

2. Gel setting occurs within approx 10 min, whereas polymerization slowly proceeds to plateau in the next 50 min. It is critical during the early period that the

polymerization cassette is kept in an upright position **on a level surface**. Moreover, the oven should not be opened in order to avoid both shaking the cassette and causing the temperature to drop.

3.6. Washing, Drying, and Storing IPGs

1. At the end of the polymerization period, disassemble the cassette by removing the clamps.
2. Insert a scalpel blade between GelBond and glass plate to loosen the foil from its support.
3. Fill a plastic box in which the gel may lay flat with 1 L of glycerol washing solution.
4. Gently peel the IPG from the mold, and transfer (upside up!) into the box containing the glycerol solution.
5. Gently shake for 1 h at room temperature.
6. Lay the gel (upside up!) on a supporting glass plate partially or completely submerged in the washing solution in order to avoid trapping air bubbles (*see* **Note 8**).
7. Set the assembly upright, approx 30 cm in front of a (cool) fan, operating at high speed. The whole area should be as dustless as possible; glycerol smudge is better collected on a tray.
8. Evaluate the progression of the drying by sensing the cooling from water evaporation on the back of the glass plate and by looking for an even and matt appearance on the front of the gel. Depending on the temperature and relative humidity, the drying step may take between 1 and 2 h, and should not be unnecessarily prolonged. It is acceptable, but not recommended to let the gels dry unattended, e.g., overnight, at room temperature.
9. For barely damp gels, the very last phase of drying may occur in the refrigerator, with the gel in an open box. If needed, the polymerized gels may be stored overnight in their cassettes at 4°C before washing.
10. The dry gels may be further processed right away or stored for later use. Dry IPGs may be kept in boxes at 4°C for several days. For longer periods of time *(22)*, they are sealed in plastic bags at –20°C.

4. Notes

1. All required stock solutions are orderly aligned on the bench. It is most helpful if their sequence corresponds to the order in which they are called for by the recipe. This is especially important for the acrylamido buffers, whose bottles look necessarily alike, whereas the pK label is in a relatively small print. It is strongly suggested that the recipe be marked with a ruler and that each chemical be put back in a box after its use. When a bottle is open, hold its cap upside down on a paper tissue. Make sure you are storing the open bottles on a flat, stable surface (like a tray): Expensive Immobiline may be easily if inadvertently spilled.
2. Positive displacement measuring devices might be more accurate than air-displacement pipets. No liquid droplets must adhere to the outside of the pipet tips; for this purpose, it is usually sufficient to touch the neck of the bottle. For a quantitative transfer of the Immobiline chemicals, and especially of the basic monomers dissolved

in an alcoholic solution, two precautions should be taken; namely, slow pipeting and rinsing the pipet tips twice with distilled water after each measurement.

3. It is not customary to prepare large batches of the limiting solutions—the storage conditions in the mixture are appropriate for none of the chemicals. However, leftover solutions might be used within a couple of days.

4. In the case where the commercial products were not available, an adequate substitution for the gel mold may be a plate of plain glass (polished and saturated with silane) together with a rubber gasket of approx 0.5-mm thickness. The latter may be cut from foils of *para*-, silicone, or nitrile rubber, should have a U-shape, and should be ~5 mm wide. Thick clamps from stationery stores may then be used, but care should be taken to apply the pressure on the gasket, not inside it.

5. A note of caution for 1-D experiments with radioactive samples. In some cases, the ink gives a strong positive signal; more commonly, the writing negatively interferes with film exposure. In these experiments, all marks should then be outside the area of sample application (where their disturbing appearance may turn into the definition of useful reference points).

6. The major points of caution will be stressed once more below. The flow between the different compartments must be unhindered by air bubbles between the two vessels of the mixer or by water droplets in the outlet. No backflow should occur. The pouring should not be too fast to allow proper mixing in the chamber and to avoid turbulence in the cassette (with a proper selection of the pressure drop). The solution should evenly flow along the hydrophilic wall of the cassette (lined with GelBond) and not fall dropwise along the hydrophobic one.

7. A forced ventilation is more appropriate to this purpose than a convection oven in order to provide even and controlled heating. It should be recalled that the selected temperature is the one allowing identical incorporation efficiency for all acrylamido buffers, which grafts into the gel matrix a pH gradient exactly matching the computed course *(23)*.

8. It is sensible to set aside some containers only for this purpose, i.e., to avoid recycling between gel washing, protein staining, and blot immunodetection. Owing to its high viscosity, 100% glycerol should be avoided as stock reagent in favor of the 87% preparation; ~12 mL of the latter may be measured in a small plastic beaker instead of in a graduated cylinder. Failure to include glycerol as a humidity-conditioning agent results in slab curling and easy peeling and tearing of the gel from its support. On washing, IPGs reswell to a different extent, depending on the pH of the matrix and on the course of the glycerol gradient.

Acknowledgment

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21

Analytical IPG-Dalt

Angelika Görg and Walter Weiss

1. Introduction

Immobilized pH †*adients (IPGs) for isoelectric focusing (IEF) were introduced in 1982 *(1)*. After experiencing initial problems in handling IPGs, a basic protocol of two-dimensional electrophoresis (2-DE) with IPGs in the first dimension, followed by horizontal or vertical SDS electrophoresis (IPG-Dalt), was established in 1988 *(2)*. Since that time, the protocol has not been changed essentially. Compared to classical 2-DE with carrier ampholytes *(3,4)*, the employment of IPG-Dalt has produced significant improvements in 2-D electrophoretic separation, permitting higher resolution and reproducibility, which was also demonstrated by an interlaboratory comparison *(5)*. Alkaline proteins, normally lost by the cathodic drift of carrier ampholyte focusing or separated by NEPHGE *(6)* with limited reproducibility, were perfectly separated under equilibrium conditions *(7)*. These features together with the high loading capacity of IPG-Dalt for micropreparative runs *(8–10)* have accelerated spot identification by microsequencing, amino acid composition analysis, and mass spectrometry.

The first dimension of IPG-Dalt, IEF, is performed in individual IPG gel strips, 3- to 5-mm wide, and cast on GelBond PAGfilm (*see* **Fig. 1**). Dry strips (Immobiline DryStrips® or laboratory-made; *see* Chapters 20 and 22) are rehydrated to their original thickness of 0.5 mm with a solution containing 8 *M* urea, 0.5% detergent, and 0.2% DTT. The rehydrated strips are then placed onto the cooling plate of an electrofocusing chamber or into the grooves of the Immobiline DryStrip kit, and sample cups are placed directly onto the surface of the gel strips. Sample load is about 50–100 μg of protein, depending on the sample and gel size. Alternatively, samples are applied by in-gel rehydration (*see* Chapters 24 and 24a). Since the degree of sample entry is critical, best
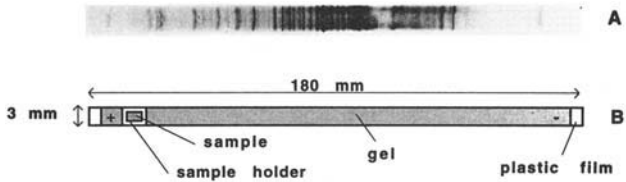
Fig. 1. (**A**) IEF in individual IPG gel strips. (**B**) Geometry of the IPG gel strip. The pH gradient and separation distance may vary by casting different IPGs and/or by using different gel lengths. Reprinted from Görg *(15)*.

Table 1
Typical Running Conditions for Analytical IPG-IEF Gel Strips *(12,13)*

Temperature	20°C		
Current max.	0.05 mA/strip, 2 mA max. in total		
Power max.	3.0–5.0 W		
Voltage max.	150 V	30 min	Sample entry
	300 V	60 min	Sample entry
	1500 V	60 min	Sample entry
	3500 V	Depending on pH gradient and separation distance *(see below)*	To the steady state
pH gradient	Separation distance 11 cm		Separation distance 18 cm
IPG 4.0–7.0	22000 Vh		42000 Vh
IPG 4.0–8.0	21000 Vh		35000 Vh
IPG 4.0–9.0	17000 Vh		30000 Vh
IPG 6.0–10.0	21000 Vh		35000 Vh
IPG 3.0–10.5	11000 Vh		18000 Vh

results are obtained using diluted samples dissolved in O'Farrell's sample solubilization buffer *(3)*, with a low voltage gradient across the gel for the first hour. The voltage is then increased to 3500 V until the steady state with constant focusing patterns is obtained. After IEF to the steady state (**Table 1**) *(11–13)*, the IPG gel strips are either equilibrated in the presence of SDS, glycerol, urea, DTT, and iodoacetamide, and immediately applied onto a horizontal or vertical SDS gel (*see* **refs.** *2,11*, and *12* and Chapters 25 and 26) or stored frozen at –78°C until used.

2. Materials
2.1. Equipment

1. Horizontal electrophoresis apparatus (e.g., Multiphor II, Amersham; Pharmacia Biotech, Uppsala, Sweden).

2. Power supply (3500 V min.) (e.g., Multidrive XL, Pharmacia Biotech).
3. Thermostatic circulator (e.g., Multitemp II, Pharmacia Biotech).
4. Glass plates with a 0.5-mm-thick U-frame (size 200 × 260 mm^2) (Pharmacia Biotech).
5. Plain glass plates (size 200 × 260 mm^2) (Pharmacia Biotech).
6. Reswelling cassette (125 × 260 mm^2) (Pharmacia Biotech).
7. Immobiline DryStrip kit (Pharmacia Biotech).
8. Parafilm.
9. Clamps (e.g., Pharmacia Biotech).
10. IEF sample applicator strip (Pharmacia Biotech).
11. Milli-Q System (Millipore, Bedford, MA).
12. Immobiline DryStrips 4-7, 3-10L, 3-10NL (Pharmacia Biotech).
13. Immobiline DrySrtip Reswelling tray (Amersham Pharmacia Biotech).

2.2. Reagents

1. IPG gel strip rehydration solution: 8 *M* urea, 0.5% CHAPS, 15 m*M* DTT, and 0.2% Pharmalyte, pH 3.0–10.0. To prepare 50 mL of the solution, dissolve 25.0 g of urea in deionized water, and complete to 50 mL. Add 0.5 g of mixed-bed ion exchanger (e.g., Amberlite MB-1), stir for 10 min, and filter. To 48 mL of this solution, add 0.25 g of CHAPS, 0.25 mL Pharmalyte, pH 3.0–10.0 (40% w/v), and 100 mg of DTT, and complete to 50 mL with deionized water. Rehydration solution should be prepared fresh the day you use it. Do not heat urea solutions above 37°C (*see* **Note 1**).
2. Sample solubilization buffer: 9.5 *M* urea, 2% CHAPS, 0.8% Pharmalyte, pH 3.0–10.0, 1% DTT, and 5 m*M* Pefabloc®. To prepare 50 mL of sample solubilization buffer, dissolve 30.0 g of urea in deionized water and make up to 50 mL. Add 0.5 g of mixed-bed ion-exchanger resin (e.g., Amberlite MB-1), stir for 10 min, and filter. Add 1.0 g CHAPS, 0.5 g DTT, 1.0 mL of Pharmalyte, pH 3.0–10.0 (40% w/v), and 50 mg of Pefabloc (Merck, Darmstadt, Germany) to 48 mL of the urea solution. Do not heat above 37°C (*see* **Note 1**). Sample solubilization buffer should always be prepared freshly. Alternatively, small aliquots (1 mL) can be stored at –78°C. Sample solubilization buffer thawed once should not be refrozen again.

3. Methods
3.1. Isoelectric Focusing (IEF)

Prior to IEF, the desired number of IPG gel strips (usually 3–4 mm wide and 180 mm long) are rehydrated in a reswelling cassette to the original gel thickness (0.5 mm). The rehydrated strips are then placed on the cooling block of the electrofocusing chamber, and samples are applied into silicone frames or sample cups using the Immobiline DryStrip kit.

1. For IEF in individual IPG gel strips, cut the dried IPG gels (*see* Chapter 20) into 3- to 4-mm-wide strips with the help of a paper cutter (*see* **Fig. 2A**). Alternatively, ready-cut IPG strips (Immobiline DryStrip) can also be used.
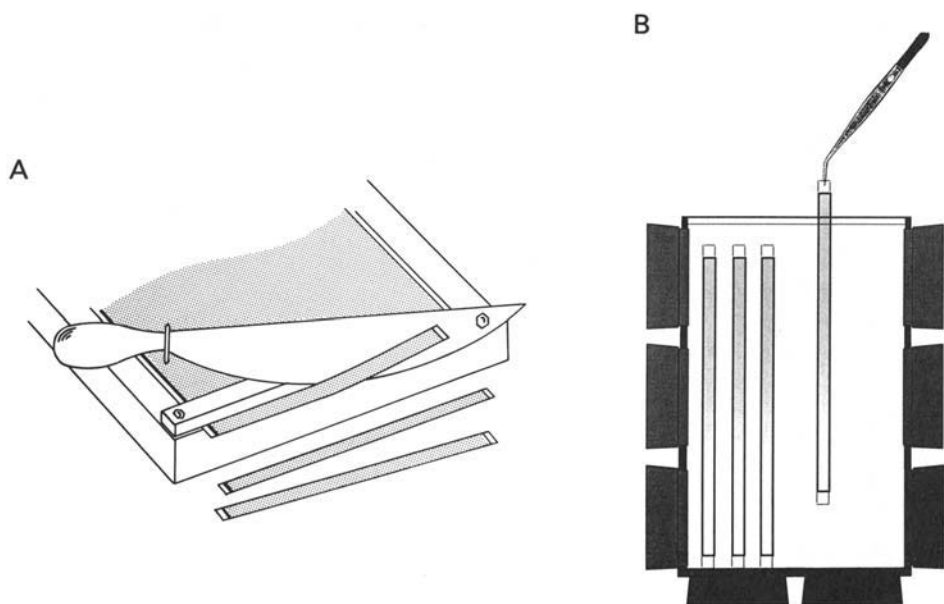
Fig. 2. **(A)** Cutting of washed and dried IPG slab gels (or Immobiline DryPlates) into individual IPG gel strips. **(B)** Rehydration of individual IPG gel strips.

2. To rehydrate the IPG gel strips to their original thickness (0.5 mm), take two layers of Parafilm, and cut out a U-frame that has the same shape and size as the U-frame attached to the glass plate. Assemble the rehydration cassette in that way that a 0.7-mm-thick U-frame (0.5-mm original thickness plus two layers of Parafilm®, 0.1 mm each) is obtained. Clamp the cassette together, and pipet in the rehydration solution.

3. Take an appropriate number of dry IPG gel strips, and pull off their protective covers. Lower them carefully, but without delay, into the rehydration cassette, gel side toward the glass plate bearing the U-frame (*see* **Fig. 2B**). Allow the strips to rehydrate overnight at room temperature. Make sure that the cassette does not leak.

4. After the IPG gel strips have been rehydrated, pour the rehydration solution out of the reswelling cassette, remove the clamps, and open the cassette with the help of a spatula. Using clean forceps, rinse the rehydrated IPG gel strips with deionized water for a few seconds and place them, gel-side up, on a sheet of water-saturated filter paper. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water, and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during IEF.

5. Cover the flat-bed cooling block with 2–3 mL of kerosene, and place the IPG gel strips (up to 40) side by side, 1–2 mm apart, on it (*see* **Fig. 3A**). The acidic ends of the IPG gel strips must face toward the anode!
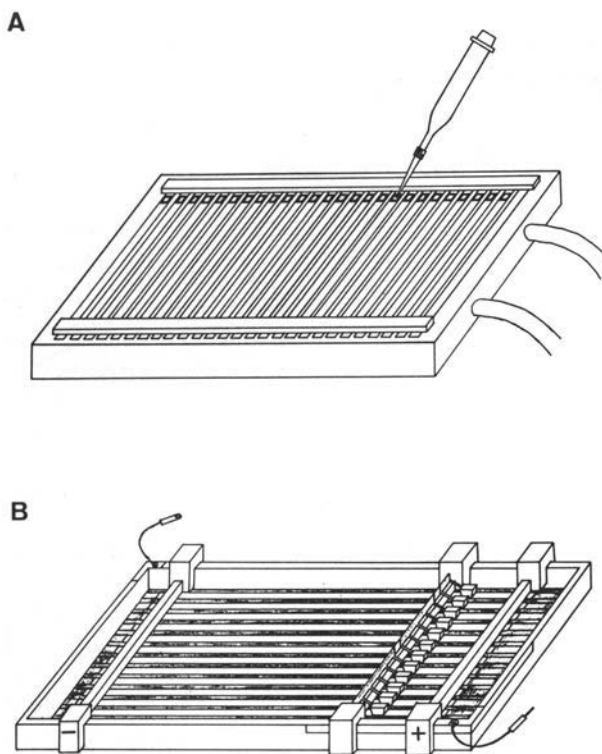
Fig. 3. IEF in individual IPG gel strips. **(A)** IPG strips are placed onto the cooling plate of the electrofocusing chamber. Samples (20 μL) are pipeted into silicone rubber frames. **(B)** IEF using the Immobiline DryStrip Kit. IPG strips are aligned in the tray, which is placed on the cooling plate. Samples (20–100 μL) are pipetted into the sample cups. The tray and the sample cups may be filled with silicone oil if pH gradient 6.0–10.0 or very narrow pH gradients (<1 pH unit) are employed.

6. Cut two IEF electrode strips (or paper strips prepared from 3-mm-thick filter paper MN 440, Macherey & Nagel, Düren, Germany) to a length corresponding to the width of all IPG gel strips lying on the cooling plate. Soak the electrode strips with deionized water, and remove excessive moisture by blotting with filter paper.

7. Place the IEF electrode strips on top of the aligned IPG gel strips at the cathode and anode. When IPGs 6–10 are used, place an additional paper strip soaked in 0.2% DTT onto the gel surface near the cathode. Paper strips soaked in 5 N NaOH may be put into to the chamber to diminish the carbon dioxide content inside.

8. Apply silicone rubber frames (2 × 5 mm^2 inner diameter) onto the gel surface, 5 mm apart from anode or cathode, for sample application (*see* **Fig. 3A**). Alternatively, sample solutions are pipeted into the sample cups provided by the Immobiline DryStrip kit (*see* Chapter 22, Subheading 3.3.1., and **Fig. 3B**).
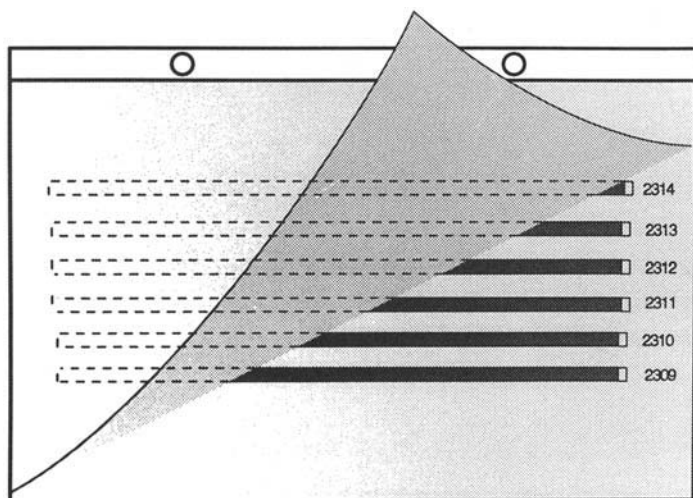
Fig. 4. Storage of the IPG gel strips. After IEF, IPG strips are stored between two plastic sheets at –78°C.

9. Pipet the samples (20 µL each, preferably dissolved in sample solubilization buffer) into the silicone frames. Protein concentration should not exceed 5–10 mg/mL. Otherwise, protein precipitation at the sample application area may occur. To avoid this, it is advantageous to dilute the sample with sample solubilization buffer and apply larger volumes instead (*see* **Note 3**).

10. Position the electrodes, and press them gently down on top of the IEF electrode strips.

11. Place the lid on the electrofocusing chamber, connect the cables to the power supply, and start IEF. Running conditions depend on the pH gradient and the length of the IPG gel strip used. An appropriate time schedule for orientation is given in **Table 1** *(12,13)*. For improved sample entry, voltage is limited to 150 V (30 min) and 300 V (60 min) at the beginning. Then continue with maximum settings of 3500 V to the steady state. Optimum focusing temperature is 20°C *(14)*.

12. After IEF, those IPG gel strips that are not used immediately for second-dimension run and (*see* Chapters 25 and 26) or are kept for further reference. For storage, the IPGs are placed between two sheets of plastic film at –78°C for up to several months (*see* **Fig. 4**).

4. Notes

1. Never heat urea solutions above 37°C in order to reduce the risk of protein carbamylation!

2. In the case of very narrow IPGs (<1 pH unit), where very long focusing times have to be applied (>200,000 Vh), it is advisable to run the IPG gel strips in the DryStrip kit covered by a protective layer of silicone oil as described above (*see* Chapter 22, Subheading 3.3.1.).

3. Sample solution should not be too concentrated to avoid protein precipitation at the sample application point. If you are in doubt, better dilute the sample with sample solubilization buffer and apply a larger volume (>20 μL) instead. In this case, Pharmacia's Immobiline DryStrip Kit® is used for sample application.

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22

IPG-Dalt of Very Alkaline Proteins

Angelika Görg

1. Introduction

Compared to classical two-dimensional electrophoresis (2-DE) with carrier ampholytes *(1,2)*, the advent of 2-DE with immobilized pH gradients (IPG-Dalt) *(3)* has produced significant improvements in 2-D electrophoretic separation with respect to:

1. Higher resolution by using well-defined narrow-pH gradients *(3)*.
2. Improved reproducibility, as was demonstrated by interlaboratory comparisons *(4,5)*.
3. Higher loading capacity for micropreparative runs, which has accelerated spot identification by microsequencing and mass spectrometry *(6–9)*.
4. The possibility to resolve—under steady-state conditions—alkaline proteins, which are normally lost by the cathodic drift of carrier ampholyte focusing or separated by nonequilibrium pH gradient electrophoresis (NEPHGE) with limited reproducibility *(10)*.

Strongly basic proteins (pI > 10), such as ribosomal and nuclear proteins, are more or less not amenable to 2-DE with carrier ampholyte isoelectric focusing (IEF) in the first dimension. Although NEpHGE *(11)* was used for the separation of ribosomal proteins and histones *(12)*, only a transient state of focusing pattern with poor resolution and limited reproducibility was obtained. Nonequilibrium focusing is difficult to control with respect to reproducibility and very sensitive to experimental conditions, batches of carrier ampholytes, run time, gel length, and sample composition *(13)*.

In contrast to former results with carrier ampholyte IEF, the use of narrow IPGs at the basic extreme should provide reproducible and highly resolved steady-state 2-D patterns of strongly alkaline proteins. However, when trying to formulate IPGs outside the pH 4.0–10.0 range, e.g., a pH 10.0–12.0 IPG interval, one is faced with severe problems because the "buffering power" of
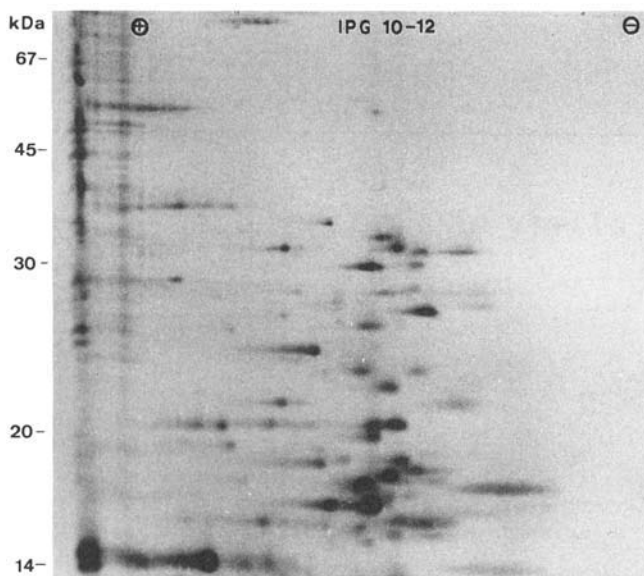
Fig. 1. IPG-Dalt of TCA/acetone extracts (primarily ribosomal proteins) from mouse liver using an IPG 10-12. IPG gels were cast with dimethylacrylamide, and the IPG strips were rehydrated in 8 *M* urea, 10% isopropanol, 10% glycerol, 1% CHAPS, 20 m*M* DTT, 0.2% Pharmalyte 3–10, and 0.2% methyl cellulose. Second dimension: vertical SDS-PAGE (13% T).

water is not negligible anymore. However, more recently, by using Immobiline pK > 13, a nonlinear IPG spanning a 10–12 interval was generated and optimized for IEF *(14,15)*, and 2-DE under steady-state conditions using IPG 10–12 and 9–12 was applied for the separation of strongly basic proteins, such as histones and ribosomal proteins *(16)* (*see* **Fig. 1**). The major changes of the new protocol compared to the standard protocol presented in Chapter 21 consist of using dimethylacrylamide instead of acrylamide and the addition of isopropanol and/or methylcellulose to the reswelling solution of the IPG strips in order to diminish the reverse electroendosmotic flow, which is very pronounced in narrow IPGs extending pH 10.0 (*see* **Table 1**) *(16)*. This is in contrast to wide IPGs, such as IPG 4–12 (Görg et al., *Electrophoresis* **19,** 1998, in press), where the standard protocol *(3)* is used.

2. Materials

2.1. Equipment

1. Electrophoresis apparatus (e.g., Multiphor II, Amersham Pharmacia Biotech).
2. Power supply (3500 V min.) (e.g., Multidrive XL, Amersham Pharmacia Biotech).
3. Thermostatic circulator (e.g., Multitemp II, Amersham Pharmacia Biotech).

Table 1
**Summary of the Major Changes of the IPG-Dalt Protocol
for Very Alkaline Proteins (pH < 12.0) Compared
to the Standard Protocol *(3)* Described for Proteins with p*I*s up to pH 10.0**

IPG-Dalt	Standard (pH < 10)	Very alkaline, pH < 12.0 narrow IPGs
IPG gel strips		
Gel matrix	Acrylamide (AA)/ *bis*-acrylamide (bis)	DMAA/*bis*-acrylamide (Bis)
Rehydration	8 *M* urea, 0.5% CHAPS 0.2 % CA, 15 m*M* DTT	8 *M* urea, 1% CHAPS, 0.2% CA 30 m*M* DTT, 10% isopropanol, 0.3% 0.3% methylcellulose or 8 *M* urea, 0.2% CA, 30 m*M* DTT, 10% isopropanol
IEF	To the steady state, at 20°C, no oil	To the steady state, at 20°C under silicone oil, argon
Storage	–78°C	–78°C
Equilibration	50 m*M* Tris-HCl, pH 8.8, 2% SDS	50 m*M* Tris-HCl, pH 8.8, 2% SDS
	1% DTT, 6 *M* urea, 30% glycerol, 4% iodoacetamide	1% DTT, 6 *M* urea, 30% glycerol, 4% iodoacetamide
SDS-PAGE	Horizontal/vertical	Horizontal/vertical

4. Immobiline DryStrip kit (Amersham Pharmacia Biotech).
5. Gradient mixer (2 × 15 mL) (e.g., Amersham Pharmacia Biotech).
6. Glass plates with a 0.5-mm thick U-frame (size 200 × 260 mm^2) (Pharmacia Biotech).
7. Plain glass plates (size 200 × 260 mm^2) (Amersham Pharmacia Biotech).
8. Reswelling cassette (125 × 260 mm^2) (Amersham Pharmacia Biotech).
9. Clamps (e.g., Amersham Pharmacia Biotech).
10. GelBond PAGfilm (200 × 260 mm^2) (e.g., FMC, Rockland, ME, or Pharmacia Biotech).
11. Heating cabinet.
12. Ultrasonic bath.
13. Magnetic stirrer.
14. Parafilm (roll, 0.5 × 15 m^2).
15. Paper electrode wicks (ultrapure) (Bio-Rad, Hercules, CA).
16. Milli-Q System (Millipore, Bedford, MA).
17. Gas cylinder filled with argon.

2.2. Reagents

1. Sample solubilization buffer: 9.5 *M* urea, 2% CHAPS, 0.8% Pharmalyte pH 3.0–10.0, 1% DTT, and 5 m*M* Pefabloc®. To prepare 50 mL of sample solubilization buffer, dissolve 30.0 g of urea in deionized water, and make up to 50 mL. Add 0.5 g of mixed-bed ion-exchanger resin (e.g., Amberlite MB-1), stir for 10 min, and filter.

Add 1.0 g CHAPS, 0.5 g DTT, 1.0 mL of Pharmalyte, pH 3.0–10.0 (40% w/v), and 50 mg of Pefabloc (Merck, Darmstadt, Germany) to 48 mL of the urea solution. Do not heat above 37°C (*see* Note 1). Sample solubilization buffer should always be prepared fresh. Alternatively, small aliquots (1 mL) can be stored at –78°C. Sample solubilization buffer thawed once should not be refrozen again.

2. Equilibration buffer: 6 *M* urea, 30% glycerol, and 2% SDS in 0.05 *M* Tris-HCl buffer, pH 8.8. To make 500 mL add: 180 g of urea, 150 g of glycerol, 10.0 g of SDS, and 50 mL of Tris-HCl buffer (0.5 *M*, pH 8.8). Dissolve in deionized water, filter, and fill up to 500 mL. The buffer can be stored at room temperature for up to 2 wk.

2.2.1. Reagents for IEF-IPG Gels

1. Acrylamide/*bis*-acrylamide solution for IPG gels: 30% T, 4% C. To make 100 mL of the solution, dissolve 28.8 g of acrylamide and 1.2 g of *bis*-acrylamide in 80 mL of deionized water. Add 0.1 g of Amberlite MB-1, stir for 10 min, filter, and fill up to 100 mL with deionized water. This solution should be prepared fresh. For IPGs 9–12 and 10–12, acrylamide is substituted by dimethylacrylamide (DMAA) (*see* **Table 2**).
2. Ammonium persulfate solution: 35% ammonium persulfate. To prepare 1 mL of the solution, dissolve 0.4 g of ammonium persulfate in 1 mL of deionized water. This solution should be prepared fresh just before use.
3. IPG gel strip reswelling solution I: 8 *M* urea, 10% (w/v) isopropanol, 1% CHAPS, 30 m*M* DTT, 0.2% Pharmalyte 3-10 (40% w/v), and 0.3% methylcellulose. Use for ribosomal proteins. To prepare 40 mL of reswelling solution I, dissolve 24.0 g of urea in 25.6 mL of deionized water and 6.3 mL isopropanol. Add 0.5 g of mixed-bed ion exchanger (e.g., Amberlite MB-1), stir for 10 min, and filter. To 39.6 mL of this solution add 0.4 g of CHAPS, 0.2 mL Pharmalyte pH 3.0–10.0 (40% w/v), 120 mg of methyl cellulose, and 160 mg of DTT. Rehydration solution should be prepared fresh the day you use it. Do not heat urea solutions above 37°C (*see* **Note 1**)!
4. IPG gel strip rehydration solution II: 8 *M* urea, 10% (w/v) isopropanol, 30 m*M* DTT, 0.2% Pharmalyte 3–10 (40% w/v), and no CHAPS. Use for IEF of histones. To prepare 40 mL of reswelling solution II, dissolve 24.0 g of urea in 25.6 mL of deionized water and 6.3 mL isopropanol. Add 0.5 g of mixed-bed ion exchanger (e.g., Amberlite MB-1), stir for 10 min, and filter. To 39.8 mL of this solution add 0.2 mL Pharmalyte, pH 3.0–10.0 (40% w/v) and 160 mg of DTT. Rehydration solution should be prepared fresh the day you use it. Do not heat urea solutions above 37°C (*see* **Note 1**)!

2.2.2. Immobiline Gel Recipes

Add reagents in order given in **Table 2**.

3. Methods
3.1. Sample Solubilization
3.1.1. Ribosomal Proteins

In order to get rid of contaminating nonribosomal proteins and of RNA molecules, preparation of ribosomes and total ribosomal proteins (Tp80S) from

Table 2
Recipes for Casting Alkaline IEF Gels with Immobilized pH Gradients[a]

Linear pH gradient	pH 8.0–12.0		pH 9.0–12.0		pH 10.0–12.0[b]	
	Heavy solution, pH 8.0	Light solution, pH 12.0	Heavy solution, pH 9.0	Light solution, pH 12.0	Heavy solution, pH 10.0	Light solution, pH 12.0
Immobiline pK 3.6	842 µL	60 µL	—	—	—	—
Immobiline pK 4.6	—	—	1030 µL	—	563 µL	—
Immobiline pK 6.2	—	—	—	—	—	—
Immobiline pK 7.0	—	—	—	—	—	—
Immobiline pK 8.5	420 µL	260 µL	—	—	—	—
Immobiline pK 9.3	107 µL	107 µL	450 µL	—	—	—
Immobiline pK 10.3	320 µL	345 µL	678 µL	380 µL	810 µL	—
Immobiline pH > 13	92 µL	400 µL	89 µL	590 µL	—	750 µL
Acrylamide/*bis* (28.8/1.2)	2.50 mL	2.50 mL	—	—	—	—
DMAA/*bis*	—	—	945 µL/30 mg	945 µL/30 mg	945 µL/30 mg	945 µL/30 mg
Deionized water	7.75 mL	11.3 mL	8.85 mL	13.1 mL	9.75 mL	13.4 mL
Glycerol (100%)	3.75 g	—	3.75 g	—	3.75 g	—
TEMED (100%)	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL
Persulfate (40%)	15.0 µL	15.0 µL	15.0 µL	15.0 µL	15.0 µL	15.0 µL
Final volume	15.0 mL	15.0 mL	15.0 mL	15.0 mL	15.0 mL	15.0 mL

[a]For effective polymerization, the solutions are adjusted to pH 7.0 with 3 N acetic acid before adding the polymerization catalysts.
[b]Recipe according to Righetti et al. (*17*), modified.

rat liver and HeLa cells according to Madjar *(18)* is recommended, comprising cell fractionation, successive removal of nuclei and mitochondriae, isolation of ribosomes followed by protein extraction, and selective RNA precipitation. Finally, the isolated ribosomal proteins (Tp80S) are reduced, alkylated, dialyzed, and lyophilized. For IPG-Dalt, the pellet is dissolved in sample solubilization buffer. Twenty microliters of sample solution containing 40 µg purified total ribosomal proteins are applied onto a single IPG strip (analytical scale).

3.1.2. Histones

Histone preparations (2 mg) are dissolved in 1 mL of sample solubilization buffer. Preparation of histones from chicken erythrocytes is according to Csordas *(19)*. Whole erythrocytes as well as isolated nuclei are extracted with 0.3 *M* HCl. Proteins are precipitated by adding the appropriate amount of trichloroacetic acid to the acid extract to give the final concentration of 25%. The protein pellets are washed with acetone and dried *in vacuo*.

3.1.3. Mammalian Cell Extracts

Mammalian cells, e.g., mouse liver, are ground in a liquid nitrogen-cooled mortar, the powder obtained is immediately suspended in 10% TCA in cold acetone ($-18°C$) containing 0.12% DTT, and kept at $-18°C$ overnight. Following centrifugation ($35,000g$, 30 min at $-10°C$), the supernatant is discarded and the pellet resuspended in acetone containing 0.2% DTT. After an hour at $-18°C$, the sample is spun again with $35,000g$ for 30 min. The supernatant is discarded and the pellet dried under vacuum. Fifteen milligrams of the pellet are suspended in 1 mL of sample solubilization buffer. After centrifugation, 20 µL/IPG strip are applied at the anode.

3.2. Gel Preparation

3.2.1. Casting Alkaline IPG-IEF Gels

Immobilized pH gradients are calculated with the help of computer assisted programs *(20,21)*. IPG gels are formed by mixing two immobiline starter solutions in a gradient mixer. All IPG gels are 0.5 mm thick and cast on GelBond PAGfilm. The mold consists of two glass plates (one covered with the GelBond PAGfilm, and the other bearing the U-frame [0.5 mm thick]) and is loaded in a vertical position. For casting IPG gels pH 10.0–12.0 and pH 9-12, acrylamide is substituted by *N,N*-dimethylacrylamide (*see* **Table 2**).

1. Prior to use, wash the glass plates thoroughly with a mild detergent and rinse with deionized water. If new glass plates are used, pipet 1–2 mL of Repel Silane® (Amersham Pharmacia Biotech) onto the glass plate that bears the U-frame, and distribute it evenly with a fuzz-free filter paper. Let it dry for a few minutes, rinse again with water, and let it air-dry. Repeat this procedure occasionally in order to prevent the gels from sticking to the glass plates.

2. To assemble the polymerization cassette (*see* **Fig. 2A**), wet the plain glass plate (size 260 × 200 mm^2) with a few drops of water. Place the Gelbond PAGfilm, hydrophilic side upward, onto the wetted surface of the plain glass plate. The GelBond PAGfilm should overlap the upper edge of the glass plate for 1–2 mm to facilitate filling of the cassette. Expel excess water with a roller. Place the glass plate that bears the U-frame (0.5 mm thick) on top of the Gelbond PAGfilm, and clamp the cassette together. Put it in the refrigerator for 30 min.

3. To cast the IPG gel, pipet 12.0 mL of the lower pH, dense solution into the mixing chamber of the gradient mixer. Outlet and valve connecting the mixing chamber and reservoir have to be closed! Add 7.5 μL of TEMED and 12 μL of ammonium persulfate, and mix. Open the connecting valve between the chambers for a second to release any air bubbles.

4. Pipet 12.0 mL of the basic, light solution into the reservoir of the gradient mixer. Add 7.5 μL of TEMED and 12 μL of ammonium persulfate, and mix with a spatula.

5. Switch on the magnetic stirrer at a reproducible and rapid rate. However, avoid excessive vortex. Remove the polymerization cassette from the refrigerator, and put it underneath the outlet of the gradient mixer. Open the valve connecting the chambers and, immediately afterward, the pinchcock on the outlet tubing so that the gradient mixture is applied slowly, but steadily into the cassette from a height of about 5 cm (*see* **Fig. 2B**). Take care that the level in both chambers drops equally fast (*see* **Note 2**). Formation of the gradient is completed in 3–5 min.

6. Keep the mold at room temperature for 15 min to allow adequate leveling of the density gradient. Then polymerize the gel for 1 h at 50°C in a heating cabinet.

7. After polymerization at 50°C, the cassette is allowed to cool down to room temperature (at least 15 min). Then insert a spatula between the glass plates and the gel, pry apart the glass plates, and carefully remove the gel from the cassette. Wash the IPG gel for 1 h with 10-min changes of deionized water (500 mL each) in a glass tray on a rocking platform. Equilibrate the gel in 2% (w/v) glycerol for 30 min, and dry it overnight at room temperature, using a fan, in a dust-free cabinet. Afterward, protect the surface of the dry gel with a sheet of plastic film. The dried IPG gel can be stored in a sealed plastic bag at –20°C for at least several months without loss of function.

3.3. Electrophoresis

3.3.1. First Dimension: IPG-IEF

Prior to IEF, the desired number of IPG gel strips (usually 3–4 mm wide and 180 mm long) is rehydrated in a reswelling cassette to the original gel thickness (0.5 mm). The rehydrated strips are then placed into the grooves of the strip aligner of the tray of the Pharmacia DryStrip Kit, and samples (up to 100 μL) are applied into sample cups placed directly on the surface of the IPG gel strips. During IEF, the IPG gel strips are protected by a layer of silicone oil.

1. For IEF in individual IPG gel strips, cut the dried IPG into 3- to 4-mm-wide strips with the help of a paper cutter (*see* **Fig. 2C**).
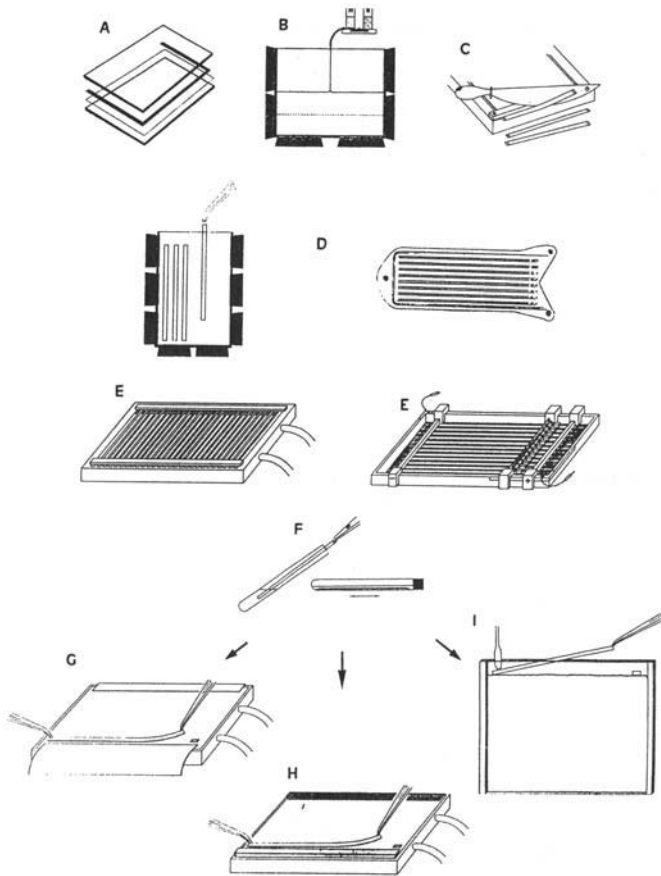
Fig. 2. Procedure of horizontal and vertical IPG-Dalt with laboratory-made and ready-made SDS gels, respectively. **(A)** Assembly of the polymerization cassette for the preparation of IPG and SDS gels cast on plastic backings (glass plates, GelBond PAGfilm, U-frame 0.5 mm thick); **(B)** casting of IPG and/or SDS pore gradient gels; **(C)** cutting of washed and dried IPG slab gels into individual IPG gel strips; **(D)** rehydration of individual IPG gel strips (left: vertical rehydration cassette; right: reswelling tray); **(E)** IEF in individual IPG gel strips (left: IPG strips directly applied on cooling late; right dry strip kit); **(F)** equilibration of IPG gel strips prior to SDS-PAGE; **(G)** transfer of the equilibrated IPG gel strip onto the surface of a laboratory-made horizontal SDS gel alongside the cathodic electrode wick; **(H)** transfer of the equilibrated IPG gel strip onto the surface of a ready-made horizontal SDS-gel alongside the cathodic buffer strip; **(I)** loading of the equilibrated IPG gel strip onto the surface of a vertical SDS gel.

2. To rehydrate the IPG gel strips to their original thickness (0.5 mm), take two layers of Parafilm and cut out a U-frame, which has the same shape and size as the glass plate's U-frame. Assemble the rehydration cassette in that way that a

0.7-mm-thick U-frame (0.5-mm original thickness plus two layers of Parafilm, 0.1 mm each) is obtained. Clamp the cassette together, and pipet in the rehydration solution (*see* **Note 3**).

3. Take an appropriate number of dry IPG gel strips, and pull off their protective covers. Lower them carefully, but without delay, into the rehydration cassette, gel side toward the glass plate bearing the U-frame (*see* **Fig. 2D**). Allow the strips to rehydrate overnight at room temperature. Make sure that the cassette does not leak (*see* **Note 3)**.

4. After the IPG gel strips have been rehydrated, pour the rehydration solution out of the reswelling cassette, remove the clamps, and open the cassette with the help of a spatula. Using clean forceps, rinse the rehydrated IPG gel strips with deionized water for a few seconds, and place them, gel-side up, on a sheet of water-saturated filter paper. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water, and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during IEF.

5. Pipet 3–4 mL of kerosene or silicone oil onto the cooling plate of the Multiphor II Electrophoresis unit, and position the Immobiline DryStrip tray on the cooling plate. Avoid trapping large air bubbles between the tray and the cooling plate.

6. Connect the electrode leads on the tray to the Multiphor II unit.

7. Pour about 20 mL of silicone oil into the tray.

8. Place the Immobiline strip aligner into the tray on top of the oil.

9. Transfer the rehydrated IPG gel strips (acidic end toward the anode) into adjacent grooves of the aligner in the tray. Align the strips such that the anodic gel edges are lined up.

10. Cut two IEF electrode strips (or paper strips prepared from 3-mm-thick filter paper MN 440, Macherey & Nagel, Düren, Germany) to a length corresponding to the width of all IPG gel strips lying in the tray. Soak the electrode strips with deionized water, and remove excessive moisture of the anodic electrode strip by blotting with filter paper. For optimum results, it is advisable to replace the electrode strips by new ones every 3 h after starting the IEF.

11. Place the moistened IEF electrode strips on top of the aligned strips near the cathode and anode.

12. Position the electrodes, and press them gently down on top of the IEF electrode strips.

13. Put the sample cups on the sample cup bar. Place the cups high enough on the bar to avoid touching the gel surface. Put the sample cup bar in position so that the sample cups are a few millimeters away from the anode.

14. Move the sample cups into position, one sample cup above each IPG gel strip, and finally press down the sample cups to ensure good contact with each strip (*see* **Fig. 2E**, right).

15. Once the sample cups are properly positioned, pour about 80 mL silicone oil into the tray so that the IPG gel strips are completely covered. The silicone oil has to be degassed and flushed with argon prior to use. If silicone oil leaks into the

Table 3
Running Conditions for Basic IPG Gels

Temperature	20°C		
Current max.	0.05 mA/strip, 2 mA max. in total		
Power max.	3.0–5.0 W		
Voltage max.	150 V	60 min	Sample entry
	300 V	60 min	Sample entry
	600 V	60 min	Sample entry
	3500 V/5000 V	Depending on pH gradient and separation distance *(see below)*	To the steady state
pH gradient	Separation distance 11 cm		Separation distance 18 cm
IPG 8–12	15000 Vh		35000 Vh
IPG 9–12	30000 Vh		45000 Vh
IPG 10–12	35000 Vh		50000 Vh

      sample cups, suck the oil out, readjust the sample cups, and check for leakage again. Add approx 150 mL of silicone oil to cover the sample cups completely.

16. Pipet the samples into the cups by underlaying. Watch again for leakage! (*See* **Note 3**.)

17. Close the lid of the Multiphor II electrophoresis chamber, and replace the atmosphere in the chamber by argon from a gas cylinder. Start IEF according to the parameters given in **Table 3**. For improved sample entry, voltage is limited to low settings (150 V) at the beginning. Then continue with maximum settings of 3500 V (110 mm separation distance) and 5000 V (180 mm separation distance) to the steady state (*see* **Table 3**). Optimum focusing temperature is 20°C *(21)*.

18. When the IEF run is completed, remove the electrodes, sample cup bar, and IEF electrode strips from the tray. Use clean forceps, and remove the IPG gel strips from the tray. Those IPG gel strips that are not used immediately for second-dimension run and/or are kept for further reference are stored between two sheets of plastic film at –78°C for up to several months (*see* Chapter 21).

3.3.2. Equilibration of the IPG Gel Strips

    The IPG gel strips are equilibrated twice, each time for 15 min in 2 × 10 mL equilibration buffer. The equilibration buffer contains 6 *M* urea and 30% glycerol in order to diminish electroendosmotic effects *(3)*, which are held responsible for reduced protein transfer from the first to the second dimension. During the second equilibration step, 260 m*M* iodoacetamide is added to the equilibration buffer in order to remove excess DTT (responsible for the "point streaking" in silver-stained patterns) *(3)*. The equilibrated IPG gel strips are slightly

blotted to remove excess equilibration buffer and then applied onto the second-dimension SDS gel.

1. Dissolve 100 mg of DTT in 10 mL of equilibration buffer (=equilibration buffer I). Take out the focused IPG gel strips from the freezer and place them into individual test tubes (200 mm long, 20 mm id). Add equilibration buffer I and 50 μL of the bromophenol blue solution. Seal the test tubes with Parafilm, rock them for 15 min on a shaker (**Fig. 2F**), and then pour off the equilibration buffer.
2. Dissolve 480 mg of iodoacetamide in 10 mL of equilibration buffer (=equilibration buffer II). Add equilibration buffer II and 50 μL of bromophenol blue solution to the test tube as above, and equilibrate for another 15 min on a rocker.
3. After the second equilibration step, rinse the IPG gel strip with deionized water, and put it on a piece of filter paper to remove excess equilibration buffer. The strip should be turned up at one edge for a few minutes to help it drain.

3.3.3. Second Dimension: SDS-PAGE

The second-dimension (SDS-PAGE) is performed horizontally or vertically *(3,10,16)*. For the horizontal setup, laboratory-made or ready-made SDS polyacrylamide gels (0.5 mm thick on GelBond PAGfilm) are placed on the cooling plate of the horizontal electrophoresis. Electrode wicks (Bio-Rad) or buffer strips from polyacrylamide (Pharmacia) are then applied. The equilibrated IPG gel strip is simply placed gel side down onto the surface of the horizontal SDS gel without any embedding procedure (*see* **Fig. 2G**). Horizontal setups are perfectly suited for the use of ready-made gels on film supports (*see* **Fig. 2H**) (Chapter 25). In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels (*see* **Fig. 2I**) and embedded in agarose (*see also* Chapter 26).

4. Notes

1. Never heat urea solutions above 37°C in order to reduce the risk of protein carbamylation!
2. When one of the chambers of the gradient mixer is emptying faster than the other during gel casting, check if there is an air bubble in the connecting line or whether the speed of the magnetic stirrer is not appropriate.
3. Alternatively, sample may be applied by in-gel rehydration (Chapter 24).

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23

Running Preparative Carrier Ampholyte and Immobilized pH Gradient IEF Gels for 2-D

Neil M. Matsui, Diana M. Smith-Beckerman, Jenny Fichmann, and Lois B. Epstein

1. Introduction

2-D gel electrophoresis is one of the most effective techniques for high-resolution separation of complex protein mixtures. Recent developments in the field of protein mass spectrometry allow the rapid and highly sensitive identification of proteins, and thus amplify the power of preparative 2-D gel electrophoresis.

In our experience, preparative IPG-IEF has several advantages compared to both carrier ampholyte (CA)-IEF and NEPHGE CA-IEF. Although good separation of proteins can be achieved by all three methods, differences caused by the mode of gradient generation cause different ranges of resolution. The traditional O'Farrell CA-IEF *(1)* is generally poor and inconsistent at resolving proteins of pI > 6.5. Horizontal streaks result from poor focusing on the neutral-to-basic end of the IEF dimension in CA-IEF. NEPHGE CA-IEF resolves basic proteins as well as acidic proteins, but owing to the lack of focusing at each protein's specific pI, the separation is also largely influenced by the size of the proteins. Thus, a large protein of pI equal to a small protein will migrate more slowly and appear to be more acidic than the small protein. Although the separation of proteins by preparative nonequilibrium pH gel electrophoresis (NEPHGE) CA-IEF is good, the reproducible localization of proteins is poor because of pI shifts and inconsistency. IPG-IEF overcomes the inherent weaknesses of CA-IEF, and NEPHGE CA-IEF because the pH gradient is immobilized. Good resolution throughout the entire pH gradient and reproducibility of protein separation is consistently achieved *(2–5)*.
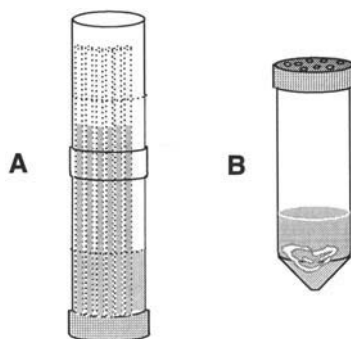
Fig. 1. Casting and equilibration of CA-IEF gels. **(A)** A casting apparatus is made by removing the conical tips of two 50-mL polypropylene centrifuge tubes and joining them by hot glue. Tubes are place in the apparatus, and gel solution is added to the apparatus. Excess space can be filled with large glass rods. To obtain the desired gel height, water is added to the apparatus. **(B)** A "salt-shaker" cover for draining equilibration buffer is created by drilling multiple small holes in caps of 50-mL polypropylene centrifuge tubes.

Here we describe both IEF methods, modified for use with large-format SDS-PAGE second-dimension gels that are compatible with subsequent protein identification using mass spectrometry. We have successfully identified many proteins from both preparative CA *(6–10)* and immobilized pH gradient (IPG) *(11)* 2-D gels loaded with up to 1 mg of total cell lysate protein. In this chapter, we also present a comparison of both CA and IPG IEF for preparative samples based on our experience with both methods.

2. Materials

2.1. Carrier Ampholyte Isoelectric Focusing (see Note 1)

1. Apparatus: a large CA first-dimension system, such as the Investigator System (ESA, Chelmsford, MA), Iso-Dalt System (Hoefer Pharmacia Biotech, San Francisco, CA), or Protean II System (Bio-Rad, Hercules, CA), including electrophoresis unit, tubes, and a gel-extruding apparatus (usually a modified syringe).
2. Power supply capable of 3500 V, preferably programmable and compatible with the electrophoresis system.
3. Casting tube and equilibration tubes: requires 50-mL polypropylene tubes, a hot glue gun, a hand drill, and scalpel or saw (**Fig. 1**). There are commercially available casting apparatuses as well.
4. CA-IEF gel solution: 8.0 *M* urea, 2% *n*-octylglucoside, 4.4% acrylamide (0.065% *bis*-acrylamide), 13.75% ampholytes (*see* **Note 2**). Degas. Polymerize with 0.117% *N,N,N',N'*-tetramethylethylenediamine (TEMED), 0.033% ammonium persulfate (APS) (or 11.7 μL TEMED and 33.3 μL 10% APS/10 mL gel solution).

5. Sample buffer CA: 8 *M* urea, 4% *n*-octylglucoside, 100 m*M* dithiothreitol (DTT), 5.5% ampholytes (*see* **Note 3**). Store at –70°C. Thaw at 37°C prior to use.
6. Sample overlay solution: 0.5 *M* urea, 0.2% *n*-octylglucoside, 0.3% sodium dodecyl sulfate (SDS), 50 m*M* DTT, 0.02% bromophenol blue. Store at –70°C. Thaw at 37°C prior to use.
7. Cathode buffer: 100 m*M* NaOH. Prepare fresh solution.
8. Anode buffer: 0.06% phosphoric acid. Prepare fresh solution.
9. Equilibration buffer: 100 m*M* Tris-HCl, pH 6.8, 0.3% SDS, 50 m*M* DTT, 0.0005% bromophenol blue (BPB), 100 m*M* sodium thioglycollate. Prepare fresh solution.

2.2. Immobilized pH Gradient Isoelectric Focusing (see Note 1)

1. Apparatus: Reswelling cassette and flatbed IEF system, such as the Multiphor system (Pharmacia Biotech, Uppsala, Sweden).
2. Power supply capable of 3500 V, preferably programmable and compatible with the electrophoresis system. The power supply must also be able to run at very low current.
3. IPG gels: 18-cm DryStrips (Pharmacia Biotech). Store at –20°C. Thaw at room temperature prior to use.
4. Reswelling buffer: 8 *M* urea, 2% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS), 65 m*M* DTT, 2% ampholytes, 1 m*M* phenyl-methylsulfonyl fluoride (PMSF), 0.0005% BPB (*see* **Note 3**). Prepare fresh solution.
5. Sample buffer IPG for cathodic sample application: 8 *M* urea, 2% CHAPS, 65 m*M* DTT, 40 m*M* Tris base, 1 m*M* PMSF, 0.0005% BPB. Store at –70°C. Thaw at 37°C prior to use.
6. Overlay: purified mineral oil (Pharmacia Biotech).
7. Equilibration buffer: 50 m*M* Tris-HCl, pH 6.8, 8.2 *M* urea, 30% glycerol, 1.0% SDS, 0.0005% BPB.

3. Methods
3.1. Preparative CA-IEF (1,12)
3.1.1. Casting the CA-IEF Tubes

1. Place glass IEF tubes in the casting apparatus (**Fig. 1A**) (*see* **Note 4**).
2. Prepare CA-IEF gel solution, degas, add polymerizing agents, and gently pour into the side of the casting apparatus. Gel solution should fill tubes by capillary action from the bottom. Add water into the side of the casting apparatus to push the gel solution to the desired height in the tubes.
3. Cover with plastic wrap, and allow to polymerize overnight at room temperature (*see* **Note 5**).

3.1.2. CA-IEF Electrophoresis (see Note 6)

1. Add anode buffer to the lower buffer chamber.
2. Remove tubes from caster, and clean them to remove excess gel. Place the tubes in the IEF apparatus. Be sure that they are snug to prevent leaks.

3. Add cathode buffer to the upper chamber to cover the tube gels. Use a long gel-loading pipet tip with a micropipetor or a Hamilton syringe to remove trapped air bubbles.
4. Pipet 30 µL of overlay buffer into each tube using a long pipet tip. Avoid the introduction of air bubbles.
5. Cover the apparatus and check for a complete circuit. Prefocus allowing the voltage to ramp to 750–800 V at a maximum current of 0.6 mA. This should take 2–4 h.
6. Dilute the sample lysate in sample buffer CA so that the 0.8–1.0 mg of protein is in a total volume of 75–200 µL (the ideal volume will depend on the empty space in the gel tube) (*see* **Note 7**).
7. After prefocusing is completed, turn the power off and open the apparatus.
8. Gently load the diluted sample lysate into tubes using a long gel-loading pipet tip or Hamilton syringe.
9. Cover the apparatus and check for a complete circuit. Focus for 17,500 V·h at a maximum current of 0.6 mA.

3.1.3. Equilibration of the CA Gels
for Use with the SDS-PAGE Second-Dimension Gels

1. Fill a tray with ice. Put 25 mL of equilibration buffer in several 50-mL tubes at room temperature.
2. After the power is turned off, open the electrophoresis chamber. Remove the tubes, and place them on the tray of ice for approx 5 min (to allow them to harden).
3. Extrude the gels from the IEF tubes into the 50-mL tubes containing equilibration buffer using a syringe filled with water affixed to the IEF tube.
4. Allow the gels to equilibrate for 15 min with shaking, then replace the tube cap with a strainer cap (**Fig. 1B**), and drain remaining buffer.
5. Add 25 mL of equilibration buffer, replace the strainer cap, with the tube cap and equilibrate for 15 min again. Drain equilibration buffer. Load the gels onto the SDS-PAGE second-dimension slab gels (*see* **Note 8**).
6. **Figure 2** shows an example of a CA-IEF 2-D gel using 1 mg of whole-cell lysate of ME-180 cervical carcinoma cells.

3.2. Preparative IPG-IEF (11,13)

3.2.1. Reswelling of IPG Strips (see **Note 9**)

1. Immobiline DryStrips are placed face-up on the reswelling cassette. A film of water can be used to help adhere the strips to the glass surface (*see* **Note 4**).
2. The cassette is closed and clamped. Reswelling buffer is injected into the cassette slowly to prevent bubbles from being trapped. A parafilm sheet should be clamped over the open end of the cassette. If desired, edges can be sealed further with agarose.
3. Place the entire cassette in a closed plastic container with a wet paper towel to prevent urea crystallization.
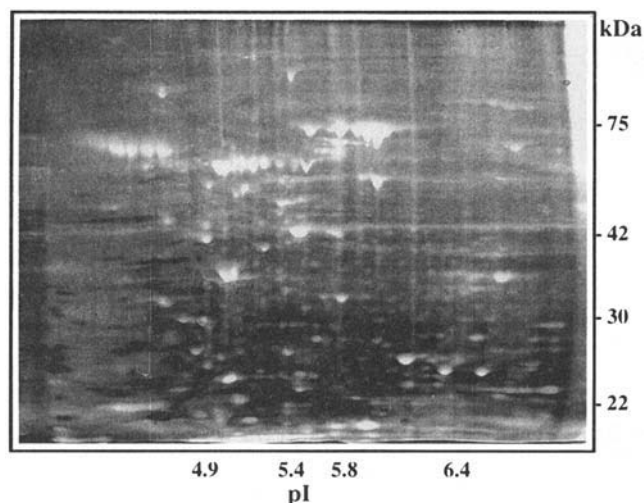4. Allow reswelling to take place overnight at room temperature.

Fig. 2. Preparative carrier ampholyte 2-D gel stained by the imidazole-zinc-negative stain. One milligram of protein from ME-180 cervical carcinoma cells was separated by carrier ampholyte IEF and 11% SDS-PAGE, and then stained using the imidazole-zinc stain. Gels were scanned by a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

3.2.2. IPF-IEF Electrophoresis (see **Note 9**)

1. One hour prior to use, be sure that the recirculating cooling unit is set to 23°C and running.
2. Apply and spread mineral oil over the cooling plate, and place the glass electrophoresis tray on the oil so that there is good contact with the cooling plate to allow even cooling.
3. Apply and spread a thin layer of mineral oil in the glass electrophoresis tray, and place the flexible plastic strip aligner on the oil. Be sure that bubbles are not trapped under the plastic tray. This will result in uneven cooling.
4. Turn the reswelling cassette over. Remove clamps, parafilm, and the top glass plate. The gel strips should be placed so that the gel side of the strips face down.
5. Remove a gel strip, and gently blot off excess reswelling buffer. Place the strip face up in the plastic strip tray (arrows facing the acid/anodic side) (*see* **Note 10**). Repeat for each gel strip.
6. After all strips are in the tray, arrange the strips so that the basic/cathodic side of the gels is aligned.
7. Wet two narrow blotter paper wicks with water; place one each on the basic/cathodic side and on the acidic/anodic side. Press the electrode bars onto the appropriate wicks. Check to make sure that the electrode bars are properly connected to the electrodes on the electrophoresis tray.

8. Place the sample cup bar (with sample cups) over the desired loading site (*see* **Note 11**).
9. Flood the electrophoresis tray with oil until all the strips and sample cups are completely covered.
10. Dilute the sample to the desired concentration (we typically use 300–1000 µg of protein in 100–200 µL) in sample buffer IPG (*see* **Notes 7** and **12**).
11. Pipet the samples into the cups. The denser sample should displace the oil in the cup.
12. Check that the circuit is complete, and firmly close the chamber.
13. Electrophoresis is performed at a maximum current of 1 mA, maximum power of 5 W, and as follows: 3 h ramp to 150 V, 4 h ramp to 300 V, 2 h ramp to 600 V, 1 h ramp to 1000 V, 9 h ramp to 3500 V (total of 35350 V·h ramp time), and 23 h at 3500 V (80500 V·h) (*see* **Note 13**).

3.2.3. Equilibration of the IPG-IEF Strips for Use with the SDS-PAGE Second-Dimension

1. After the power is turned off, open the chamber and remove the electrode and sample cup bars. Gently remove the wicks so as not to disturb the gel strips.
2. Remove each strip and gently blot off the excess oil. The strips should be placed in Pyrex 25 × 200-mm screw-capped tubes. Alternatively, strips can be placed between acetate sheet protectors. The strips can be stored at –70°C until used (*see* **Note 14**).
3. Prior to the second-dimension run, strips are equilibrated for 10 min with shaking in equilibration buffer plus 2% DTT, followed by 10 min with shaking in equilibration buffer plus 2.5% iodoacetamide (*see* **Note 8**).
4. **Figure 3** shows an example of an IPG-IEF 2-D gel using 1 mg of ME-180 R31 whole-cell lysate.

4. Notes

1. Solutions should be prepared in clean glassware with deionized, distilled water. All solutions containing urea should be made fresh (unless indicated otherwise) and filtered (1.2-µ filter pore size) after preparation to prevent undesired urea degradation products and urea crystallization. In general, reagents for IEF should be electrophoresis grade.
2. We use Duracryl (ESA) in place of acrylamide to prepare tube gels for CA-IEF. In our experience, Duracryl gels are less prone to breakage than acrylamide gels during extrusion.
3. CHAPS and *n*-octylglucoside have not been found to interfere with mass spectrometry. Detergents that tightly associate with proteins and have repeating components, such as NP40, should not be used if mass spectrometry is used to analyze 2-D gel-separated proteins.
4. Solutions and gels should be handled with gloves, and all steps carried out at room temperature unless otherwise noted.
5. Tube gels may develop urea crystals with cold overnight temperatures—investigators with such conditions may wish to leave the casting apparatus in a 20–25°C water bath during polymerization.
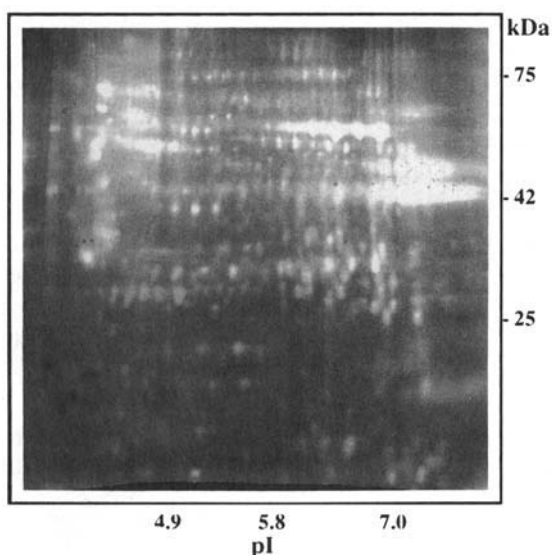
Fig. 3. Immobilized pH gradient 2-D gel stained by the imidazole-zinc-negative stain. One milligram of protein from ME-180 cervical carcinoma cells was separated by immobilized pH gradient (3.0–10.0 nonlinear) IEF and 11% SDS-PAGE, and then stained using the imidazole-zinc stain. Gels were scanned by a Computing Densitometer (Molecular Dynamics).

6. We have performed NEPHGE *(14)* modified for preparative electrophoresis to separate basic proteins using a carrier ampholyte system on the Investigator system (ESA). Briefly, no prefocusing is performed. Cathode buffer is added to the lower buffer chamber, and anode buffer is added to the upper buffer chamber. The protein sample is loaded on the anodic/acid side of the gel. The electrophoresis starts with ramping from 0–1000 V over 4–7 h at 0.6 mA maximum current, and then for 4000 V·h at 0.6-mA maximum current.

7. The same procedures can be used in preparation for 2-D gel immunoblots from complex protein samples. We typically use 200–500 μg of protein for 2-D gel immunoblots.

8. After equilibration, the CA-IEF tubes are laid on the stacking gel of a large format SDS-PAGE gel, or the IPG-IEF strips are laid on large-format SDS-PAGE gels and overlaid with 0.5% agarose in 50 m*M* Tris, pH 6.8. Both the Investigator system (ESA) and Iso-DALT system (Hoefer Pharmacia Biotech) have been successfully used.

9. Since the original writing of this manuscript, we have modified our procedure for use with the new reswelling cassettes, which have slots to reswell gels individually (Hoefer Pharmacia Biotech). Briefly, in lieu of **steps 1–3** in **Subheading 3.2.1.**, each slot is filled with 400 μL of sample in reswelling buffer. Avoid putting buffer in the wells at the end of the slot. Place an Immobiline DryStrip in

each slot laying gel side down on the sample. Overlay each IPG strip with 1–2 mL of mineral oil. **Steps 4**, **8**, **10**, and **11** of **Subheading 3.2.2.** may be omitted, and each gel strip can be removed from the cassette and the excess oil blotted at that point.

10. It is important to do this step in a timely fashion to prevent urea crystallization.

11. The optimal sample application site (i.e., acidic or basic pH) varies with the sample. We have found that loading on the basic end works well for whole-cell lysates of A375 human melanoma cells and ME-180 human cervical carcinoma cells.

12. The sample buffer used may be dependent on which part of the gradient the sample is loaded—an alternate buffer is: 8 *M* urea, 2% CHAPS, 65 m*M* DTT, 0.8% ampholytes, 1 m*M* PMSF, 0.0005% bromophenol blue.

13. If resolution along the IEF dimension is unsatisfactory, it may be advisable to increase the ramp time and the focusing V·h. Also note that IPG IEF runs at very low current.

14. We have stored strips at –70°C for up to 2 mo and noticed no alterations in the IEF resolution.

Acknowledgments

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24

In-Gel Sample Rehydration
of Immobilized pH Gradient

Jean-Charles Sanchez, Denis Hochstrasser, and Thierry Rabilloud

1. Introduction

Two-dimensional gel electrophoresis (2-DE) is a useful proteomic tool to unravel some of organism gene expression complexities, and to study directly the gene products and several of their corresponding posttranslational modifications. A simple and universal sample application method was developed *(1,2)* to provide reproducible separation of thousands of polypeptides on a single gel and their transfer to PVDF membrane. This methodology is suitable for commercially available or homemade IPG strips. Practically, we combined "volume-controlled rehydration" and "in-gel sample rehydration," using the entire IPG gel for sample application, with the protein entering the gel during its rehydration. This method avoids the use of sample cups, eliminates precipitation at the sample application point, and thus, improves resolution throughout the pH range of the gel. It also allows precise control of protein amounts and sample volumes loaded into the IPG gels. However, the separation of low-copy-number proteins in amounts sufficient for postseparation analysis continues to present a challenge for 2-D techniques, and prefractionation techniques are still required in this situation.

2. Materials
2.1. Equipment

1. Mixer, such as "Vortexer."
2. Centrifuge.
3. DryStrip Reswelling Tray: Constructed from a block of methacrylate that accommodates IPG strips in separate grooves. Each groove was 10 mm deep, 4.0 mm
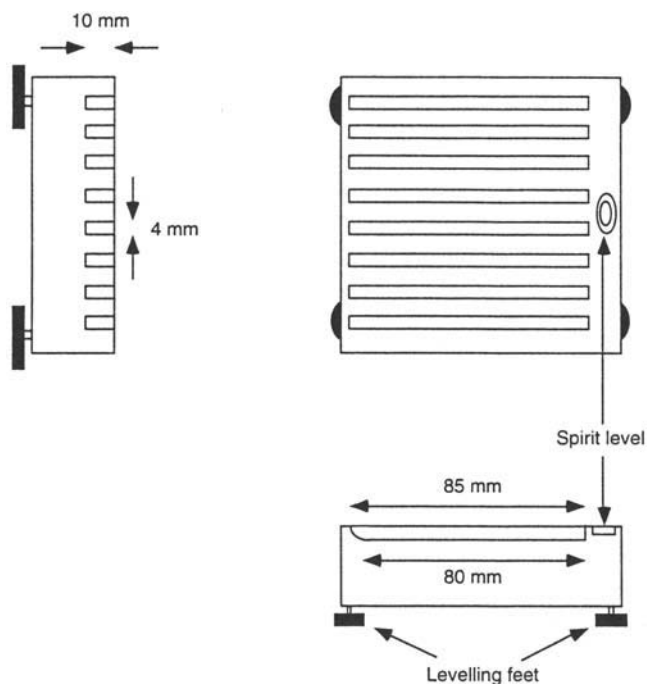
Fig. 1. A schematic diagram of the methacrylate reswelling chamber design for 110-mm IPG strips. Each groove is 10 mm deep, 4.0 mm wide, and 130 mm long. Four leveling feet and a spirit level are included in the chamber.

wide, and 85, 130, or 205 mm long according to strip length. Four leveling feet and a spirit level are included in the chamber. They are also commercially available from Pharmacia-Amersham (Uppsala, Sweden) (*see* **Fig. 1**).
4. Pharmacia strip tray.
5. Power supply (e.g., Multidrive XL, Pharmacia-Amersham).
6. Horizontal electrophoresis apparatus (Multiphor II, Pharmacia-Amersham).
7. Thermostatic circulator (Multitemp II, Pharmacia-Amersham).

2.2. Reagents

1. Rehydration solution A: 8 M urea, 4% (w/v) CHAPS, 2% (v/v) Resolytes 4-8, 65 mM DTE, and a trace of bromophenol blue.
2. Rehydration solution B: 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v), sulfobetaine 3-10, 1% (v/v) Pharmalytes 3-10.
3. Low-viscosity paraffin oil.
4. Electrode wicks.
5. Kerosene.

3. Method

3.1. Sample Application by In-Gel Rehydration

1. Cut the dried IPG gels into 3-mm-wide strips with the help of a paper cutter (*see* **Note 1** and Chapters 20 and 22). Alternatively, ready-cut IPG strips 70, 110, or 180 mm (Immobiline DryStrips) can also be used.
2. Solubilize the protein sample (0.1–5 mg) with either the rehydration solution A or B to a final volume of 400–500 μL for 180-mm strips, 230–300 μL for 110 mm strips, and 130–180 μL for 70-mm strips.
3. Level the reswelling tray.
4. Pipet the entire sample-containing rehydration solution into the grooves of the rehydration tray (*see* **Note 2**).
5. Peel off the protective cover sheets from the IPG strips, and position them such that the gel of the strip is in contact with the sample (gel-side-down).
6. Cover the IPG strips and the samples with low-viscosity paraffin oil and let the strips rehydrate from 6 h to overnight at room temperature (*see* **Note 3**).

3.2. Rehydrated IPG Positioning

1. Remove the rehydrated IPG gels carrying the protein sample from the grooves with tweezers, rinse them with deionized water, and place them, gel-side up, on a sheet of water-saturated filter paper.
2. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess of deionized water (*see* **Note 4**).
3. Cover the flatbed cooling block with 2–3 mL of kerosene, and place the Pharmacia strip tray according to the manufacturer's instructions. Position the IPG gel strips side by side. The acidic end of the IPG gel strips must face toward the anode.
4. Cut two IEF electrode wicks per strip of 1.5-cm length. Soak the electrode wicks with deionized water, and remove excessive moisture by blotting with filter paper. Place the IEF electrode wicks longitudinally on top of the aligned IPG gel strips at the cathodic and anodic gel ends with 3–5 mm overlap with the strip (*see* **Note 5**).
5. Assemble electrodes, and cover the entire setup with low-viscosity paraffin oil.

3.3. Running Conditions

Place the lid on the electrofocusing chamber, connect the cables to the power supply, and start IEF. Running conditions depend on the pH gradient and the length of the IPG gel strip used. An appropriate time schedule for orientation is given here.

3.3.1. 70-mm IPG Running Conditions

Migrate the strips as followed:

1. Increase the voltage linearly from 300 to 3500 V during 10 min.
2. Increase the voltage to 3500 V up to a total volt-hour product of 3.7 kVh.
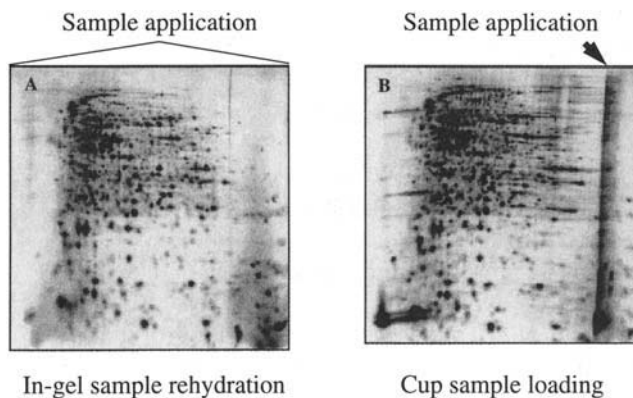
Fig. 2. Two silver-stained mini-2-D PAGE gels of *Escherichia coli* using commercial 7-cm IPG strips (3.5-10, NL Pharmacia-Amersham, Uppsala, Sweden) and home-made Hoefer (12.5% T/2.6% C, PDA) slab gels. **(A)** 20 µg of *E. coli* were loaded using in-gel sample rehydration, and **(B)** 20 µg using cathodic cup loading.

3.3.2. 110-mm IPG Running Conditions

Migrate the strips as followed:

1. Increase the voltage linearly from 300 to 3500 V during 1 h.
2. Let the voltage run for an additional hour at 3500 V.
3. Increase the voltage to 5000 V up to a total volt-hour product of 20 kVh.

3.3.3. 180-mm IPG Running Conditions

Migrate the strips as followed:

1. Increase the voltage linearly from 300 to 3500 V during 3 h.
2. Let the voltage 3 additional hours at 3500 V.
3. Increase the voltage to 5000 V up to a total volt-hour product of 50 kVh.

4. Notes

1. The critical point for correct sample absorption by the strip is the final acrylamide percentage. It should be in the range 2.5–3.5% acrylamide. Below 2.5%, water extrusion occurs, decreasing the quality of the 2-DE pattern. Above 3.5%, poor penetration of the proteins is experienced. This final acrylamide percentage can be obtained by casting a low-percentage gel rehydrated at its original thickness *(1)*. More conveniently, profit can be taken from the overswelling properties of immobiline gels *(3)*. In this case, any type of strips, including commercial ones, can be successfully used *(2)*. The final acrylamide percentage (%T) is given by the relationship: final %T × rehydration volume = casting volume × casting %T.

In the case of commercial strips, the casting volumes (in microliters) are: length (in mm) × 0.5 mm (thickness) × 3.3 mm (width) and the casting %T is 4%. Commercial 70-, 110-, and 180-mm IPG gels therefore have casting volumes of 115, 180, and 300 μL, respectively. Rehydration volumes of 170, 280, or 450 μL, respectively, correspond to a final %T of 2.6%; 3% T is obtained by rehydration in 150, 240, or 400 μL, respectively. These volumes are completely absorbed by the strips in 6 h.

2. Pipet the solution slowly to minimize the introduction of air bubbles. Remove any large bubbles that may be trapped in the solution.
3. The low-viscosity paraffin oil prevents evaporation and urea crystallization.
4. The strips are rinsed to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during IEF.
5. The electrode wicks act as a trap for ionic compounds.
6. Technically, the loading of samples by reswelling does not suffer from precipitation problems, and, therefore, should offer a means of quantitative sample loading (*see* **Fig. 2**).
7. Equilibration of the IPG strips between first and second dimension can be achieved in the rehydration tray. The design of the chamber thus allows smaller volumes to be used in separate grooves during strip equilibration, thus eliminating any crosscontamination of samples and decreasing the running cost.
8. The in-gel sample rehydration methodology avoids the use of sample cups and thus eliminates sample leakage at the sample application point (*see* **Fig. 1**).

Acknowledgments

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24A

High-Resolution, IPG-Based, Mini Two-Dimensional Gel Electrophoresis

Jean-Charles Sanchez and Denis F. Hochstrasser

1. Introduction

   The reproducibility and resolving power of immobilized pH gradient (IPG) led two-dimensional gel electrophoresis (2-DE) technology to the heart of proteome projects. Despite the recent progress achieved in this field, 2-DE is still not widely used as a screening tool either in industry or in clinical chemistry laboratories. This is mostly owing to the fact that this methodology is complex, expensive, and slow. Actually, the whole process for a standard 2-DE (160 × 180 mm) requires three to four working days depending on which procedure is used. In order to reduce significantly the above limiting factors, a mini–2-DE method was developed that has the ability to produce several protein maps easily within one working day (<6 h) at a much lower price. It uses a combination of in-gel sample rehydration *(1,2)*, small nonlinear 3.5–10 IPG gels (7 cm), homemade or precast vertical slab gels, and an automated protein-staining device. Examples of plasma and *Escherichia coli* protein separation are presented in this chapter. It demonstrates that this method is rapid, simple to perform, and keeps the advantage of the 2-D resolving power. Therefore, it opens the prospect of high 2-DE throughput for sample screening.

2. Materials
2.1. Equipment

1. Mixer such as "Vortexer."
2. Centrifuge.
3. DryStrip Reswelling Tray: Constructed from a block of methacrylate that accommodates IPG strips in separate grooves. Each groove is 10 mm deep, 4.0 mm
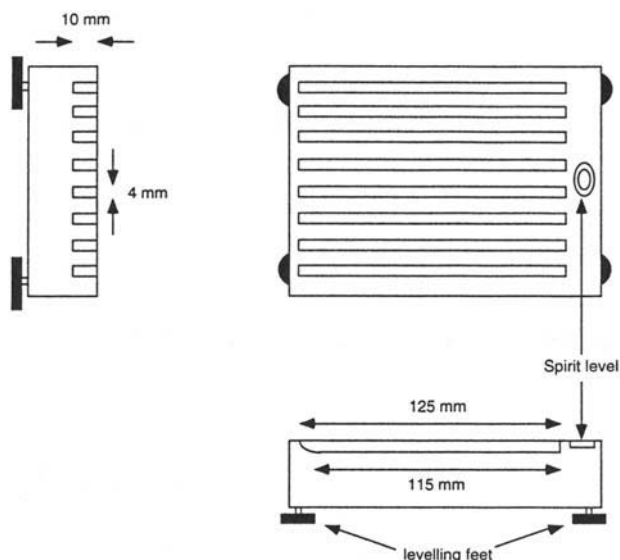
Fig. 1. A schematic diagram of the methacrylate reswelling chamber. Each groove is 10 mm deep, 4.0 mm wide, and 80 mm long. Four leveling feet and a spirit level are included in the chamber.

    wide, and 80 mm long. Four leveling feet and a spirit level are included in the
    chamber (*see* **Fig. 1**).
 4. Pharmacia strip tray.
 5. Power supply (e.g., Multidrive XL, Pharmacia-Amersham, Uppsala, Sweden).
 6. Horizontal electrophoresis apparatus (Multiphor II, Pharmacia-Amersham).
 7. Thermostatic circulator (Multitemp II, Pharmacia-Amersham).
 8. Vertical electrophoresis units (Hoefer's Mighty small SE/250 or Bio-Rad's Mini
    Protean II Cell, Hercules, CA).
 9. Automated gel stainer (Hoefer, San Francisco, CA).

2.2. Reagents

 1. Rehydration solution A: 8 *M* urea, 4% (w/v) CHAPS, 2% (v/v) Resolytes 4-8, 65 m*M*
    DTE, and a trace of bromophenol blue (*see* **Note 1**).
 2. Immobilized pH gradient 3.5–10 NL 70 mm (Pharmacia-Amersham) (*see* **Note 2**).
 3. Low-viscosity paraffin oil.
 4. Electrode wicks.
 5. Kerosene.
 6. Solution B: 50 m*M* Tris-HCl, pH 6.8, 6 *M* urea, 30% (v/v) glycerol, 2% (w/v)
    SDS, and 2% (w/v) DTE.
 7. Solution C: 50 m*M* Tris-HCl, pH 6.8, 6 *M* urea, 30% (v/v) glycerol, 2% (w/v)
    SDS, 2.5% (w/v) iodoacetamide, and a trace of bromophenol blue.

8. Solution D: 0.5% agarose, 25 m*M* Tris, 192 m*M* glycine, and 0.1% SDS.
9. Solution E: 25 m*M* Tris, 192 m*M* glycine, and 0.1% SDS.
10. 30% Acrylogel-PIP 26 (BDH).
11. Solution F: 1.5 *M* Tris-HCl, pH 8.8.
12. 10-20% Ready 2-D gels (Bio-Rad).
13. 1 *M* Sodium thiosulfate.
14. 10% Ammonium persulfate.
15. TEMED

3. Method

3.1. Sample Application by In-Gel Rehydration

1. Twenty micrograms of any mixture of proteins *(3)* are mixed with 150 µL of rehydration solution A (*see* **Note 1**). The whole final diluted sample is used for in-gel sample rehydration *(1,2)*.
2. Cut the dried IPG gels into 3-mm wide strips with the help of a paper cutter (*see* Chapters 20 and 22). Alternatively, 70-mm ready-cut IPG strips (Immobiline DryStrips) can be used (*see* **Note 2**).
3. Level the reswelling tray (*see* **Fig. 1**).
4. Pipet the entire sample-containing rehydration solution into the grooves of the rehydration tray (*see* **Note 2** of Chapter 24).
5. Peel off the protective cover sheets from the IPG strips, and position them such that the gel of the strip is in contact with the sample (gel-side down).
6. Cover the IPG strips and the sample with low-viscosity paraffin oil, and let the strips rehydrate from 6 h to overnight at room temperature (*see* **Note 3** of Chapter 24).

3.2. IEF Separation

1. Remove the rehydrated IPG gels carrying the protein sample from the grooves with tweezers, rinse them with deionized water, and place them, gel-side up, on a sheet of water saturated filter paper.
2. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water, and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess of deionized water (*see* Note 4 of Chapter 24).
3. Cover the flatbed cooling block (Multiphor II) with 2–3 mL of kerosene, and place the Pharmacia strip tray according to the manufacturer's instructions. Position the IPG gel strips side by side. The acidic end of the IPG gel strips must face toward the anode.
4. Cut 2 IEF electrode wicks/strip of 1.5 cm length. Soak the electrode wicks with deionized water, and remove excessive moisture by blotting with filter paper. Place the IEF electrode wicks longitudinally on top of the aligned IPG gel strips at the cathodic and anodic gel ends with 3–5 mm overlap with the strip (*see* Note 5 of Chapter 24).
5. Cover the entire setup with low-viscosity paraffin oil.
6. Place the lid on the electrofocusing chamber, connect the cables to the power supply, and start IEF.
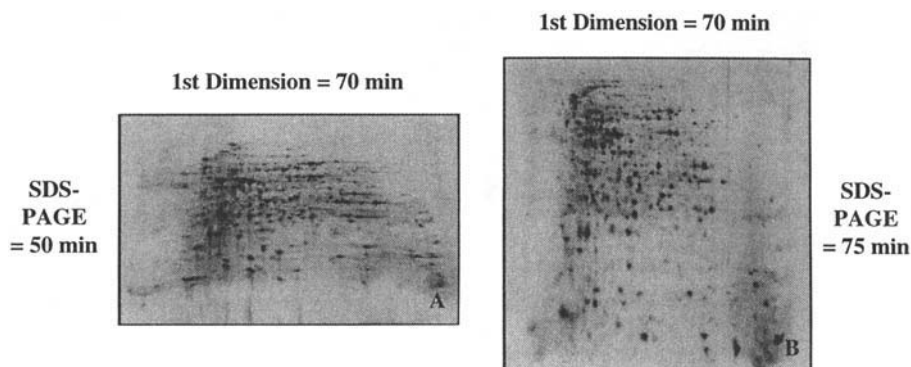
1st Dimension = 70 min

1st Dimension = 70 min

SDS-
PAGE
= 50 min

SDS-
PAGE
= 75 min

Fig. 2. Two "mini"–silver-stained 2-D PAGE gels of *E. coli* (20 μg) using 7-cm IPG strips (3.5-10, NL Pharmacia-Amersham). **(A)** Ready 2-D Gel (10-20% T, Bio-Rad) is used for the 2-D separation, or a **(B)** homemade Hoefer (12.5% T/2.6% C, PDA) slab gel.

7. Increase the voltage linearly from 300 to 3500 V during 10 min, followed by one additional hour at 3500 V.
8. Equilibrate the strips for 12 min in the rehydration tray using 3 mL/groove of equilibration solution B and then for 5 min using equilibration solution C (*see* **Note 3**).

3.3. SDS-PAGE as Second Dimension

In the second dimension, precast or homemade vertical SDS slab gels can be used (*see* **Fig. 2**).

3.3.1. Bio-Rad Ready Gels

1. After the equilibration, cut the IPG gel strips to size.
2. Overlay the Bio-Rad Ready 2-D gradient gels (4-20 or 10-20% T) with solution D heated at about 70°C (*see* **Note 4**).
3. Immediately load the IPG strip through it, and let the agarose solution to cool at room temperature for 5 min.
4. Assemble the electrophoresis unit (mini Protean II Cell) according to the manufacturer's instructions, and add running solution E in the upper and lower buffer chambers.
5. Apply 200 V (constant) for 50 min.

3.3.2. Homemade Gels

1. Gently mix and degas 42 mL of 30% Acrylogel-PIP 26, 25 mL of solution F, 500 μL of 1 *M* sodium thiosulfate, 10% 350 μL ammonium persulfate, 50 μL TEMED, and 32.6 mL of distilled water (*see* **Notes 5–7**) *(4,5)*.
2. Pour the mixture between the assembled 1-mm spacers and glass plates (fitting the Mighty small SE/250 electrophoresis unit from Hoefer) until 0.7 cm from the top of the plates and overlayered with sec-butanol for about 30 min. Replace the overlay by solution E until gel utilization.

Table 1
Automated, Sensitive Ammoniacal Silver Detection Method

Step		Solutions	Time
1.	Washing	Distilled water	5 min
2.	Fixation	40% (v/v) Ethanol	20 min
		10% (v/v) Acetic acid	
3.	Washing	Distilled water	5 min
4.	Sensitizing	2% (v/v) Glutaraldehyde (50%)	20 min
		0.5 *M* Sodium acetate	
5.	Washing 1	Distilled water	3 × 5 min
6.	Washing 2	0.05% (w/v) 2,7 Naphtalene disulfonic acid	2 × 20 min
7.	Washing 3	Distilled water	4 × 10 min
8.	Silver reaction	0.8% (w/v) Silver nitrate	20 min
		1.3% Ammonia (25%)	
		20 m*M* Sodium hydroxide	
9.	Washing	Distilled water	4 × 4 min
10.	Developing	0.01% (w/v) Citric acid	1–5 min
		0.1% (v/v) Formaldehyde (37%)	
11.	Stopping	5% (w/v) Tris base	10 min
		2% (v/v) Acetic acid	
12.	Washing	Distilled water	3 × 5 min
Total			210 min

3. After IPG equilibration, cut the strips to size.
4. Overlay the 12.5% T/2.6% C polymerized gels with solution D heated at about 70°C (*see* **Note 4**).
5. Load immediately the IPG strip through it, and let the agarose solution to cool at room temperature for 5 min.
6. Assemble the electrophoresis unit (Mighty small SE/250) according to the manufacturer's instructions, and add running solution, E in the upper and lower buffer chambers.
7. Apply 200 V (constant) for 1 h and 15 min.

3.4. Automated Silver Detection

1. At the end of the run, turn off the power, disassemble the gel apparatus, and stain the gels as described in Chapters 31–38 or use the automated gel-stain protocol listed below.
2. Create a protocol program, place the gels in the tray of the Hoefer automated gel stainer, and start the protocol as described by the manufacturer using a volume of 375 mL. **Table 1** describes an automated sensitive ammoniacal silver detection method *(6)* used in **Figs. 2** and **3**.
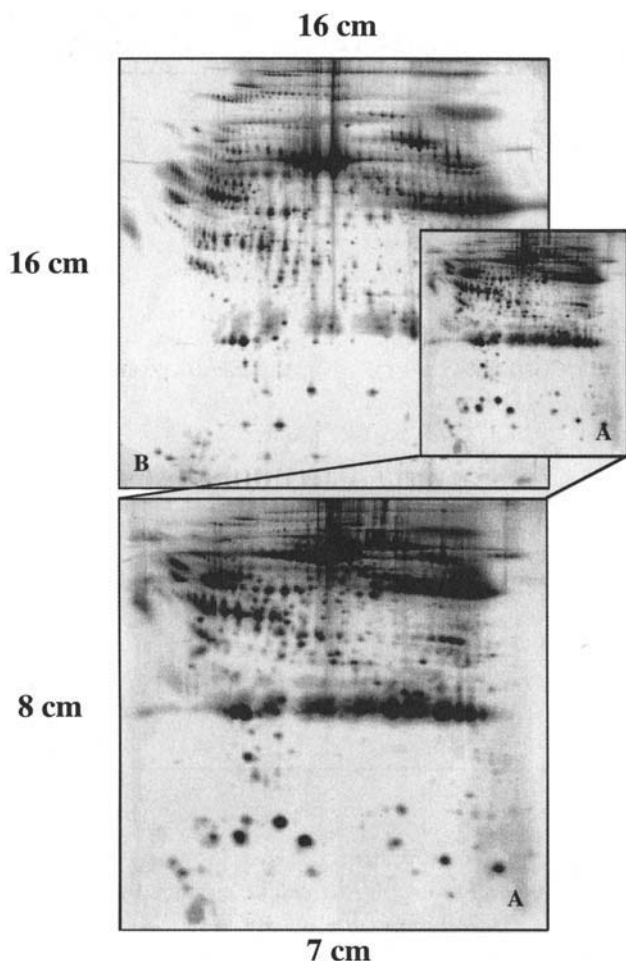3. Digitize the gels and store the images as described in Chapter 39.

Fig. 3. This shows "mini"- and "large" silver-stained 2-D PAGE gels of human plasma proteins. **(A)** 15 µg of plasma were loaded on 7-cm IPG strips (3.5–10, NL Pharmacia-Amersham) and homemade Hoefer (12.5% T/2.6% C, PDA) slab gel. **(B)** 45 µg of plasma were loaded on 18-cm IPG strips (3.5-10 NL Pharmacia-Amersham) and homemade Bio-Rad (9–16% T/2.6% C, PDA) vertical gradient slab gel.

4. Notes

1. Sample preparation and solubilization are crucial factors for the overall performance of the mini 2-D-PAGE technique. Protein complexes and aggregates should be completely disrupted in order to avoid appearance of new spots owing to a partial protein solubilization.
2. See Chapter 24 for the in-gel sample rehydration methodology.

3. After the first-dimensional separation, the strips are equilibrated in order to resolubilize proteins and reduce and alkylate disulfide bonds.

4. The combination of the IPG strip and agarose avoids the need for a stacking gel.

5. Slab gels are not polymerized in the presence of SDS. This seems to prevent the formation of micelles that contain acrylamide monomer, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerized monomer in the polyacrylamide. The SDS used in the gel running buffer is sufficient to maintain the necessary negative charge on proteins.

6. Piperazine diacrylyl (PDA) is used as crosslinker. We believe this reduces N-terminal protein blockage, gives better protein resolution, and reduces diamine silver-staining background.

7. Sodium thiosulfate is used as an additive to reduce background in the silver staining of gels.

Acknowledgments

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25

Horizontal SDS-PAGE for IPG-Dalt

Angelika Görg and Walter Weiss

1. Introduction

   After IEF-IPG (*see* Chapters 21–24a), the IPG gel strips are equilibrated in the presence of SDS, DTT, urea, glycerol, and iodoacetamide, and placed onto the surface of a horizontal or on top of a vertical SDS gel *(1–3)* (*see* Chapter 26). For horizontal setups, the laboratory-made or ready-made SDS-PAGE gel (ExcelGel SDS), cast on plastic backing, is placed onto the cooling plate of a horizontal electrophoresis system. The equilibrated IPG gel strip(s) is (are) transferred gel-side down onto the surface of the SDS gel alongside the cathodic electrode wick. For vertical setups, the equilibrated IPG gel strips are loaded on top of vertical SDS polyacrylamide gels and embedded in agarose (*see* Chapter 26). On completion of electrophoresis, the resolved polypeptides are either stained with Coomassie blue or silver nitrate, or are transferred onto an immobilizing membrane and detected with specific reagents, such as lectins or antibodies. Alternatively, they can be subjected to amino acid (sequence) analysis or mass spectrometry *(4–8)*.

2. Materials
2.1. Equipment

1. Horizontal electrophoresis apparatus (e.g., Multiphor II, Amersham Pharmacia Biotech, Uppsala, Sweden).
2. Power supply (1000 V min) (e.g., Macrodrive I, Amersham Pharmacia Biotech).
3. Thermostatic circulator (e.g., Multitemp II, Amersham Pharmacia Biotech).
4. Gradient mixer (2 × 15 mL) (Amersham Pharmacia Biotech).
5. Glass plates with a 0.5-mm-thick U-frame (size 200×260 mm^2) (Amersham Pharmacia Biotech).
6. Plain glass plates (size 200×260 mm^2) (Amersham Pharmacia Biotech).

7. Clamps (e.g., Amersham Pharmacia Biotech).
8. GelBond PAGfilm (200×260 mm^2) (e.g., FMC, Rockland, ME or Amersham Pharmacia Biotech).
9. Heating cabinet.
10. Magnetic stirrer.
11. Parafilm.
12. Paper electrode wicks (ultrapure) (Bio-Rad, Hercules, CA).

2.2. Reagents

1. Equilibration buffer: 6 *M* urea, 30% glycerol, and 2% SDS in 0.05 *M* Tris-HCl buffer, pH 8.8. To make 500 mL add: 180 g of urea, 150 g of glycerol, 10.0 g of SDS, and 50 mL of Tris-HCl buffer (0.5 *M*, pH 8.8). Dissolve in deionized water, filter, and fill up to 500 mL. The buffer can be stored at room temperature up to 2 wk.
2. Equilibration buffer I: 1% w/v DTT in equilibration buffer. To make, dissolve 100 mg of DTT in 10 mL of equilibration buffer. Prepare fresh each time.
3. Equilibration buffer II: 4.8% w/v iodoacetamide in equilibration buffer. To make, dissolve 480 mg of iodoacetamide in 10 mL of equilibration buffer. Prepare fresh each time.
4. Bromophenol blue solution: 0.25% bromophenol blue. To make 10 mL: dissolve 25 mg of bromophenol blue in 10 mL of Tris-HCl buffer (0.5 *M*, pH 8.8) buffer. Store at 4°C.
5. Molecular mass (M_r) marker proteins (e.g., Bio-Rad or Pharmacia-Biotech).
6. Stacking gel buffer: 0.5 *M* Tris-HCl, pH 6.8, and 0.4% SDS *(9)*. To make 100 mL, dissolve 6.05 g of Trizma base, 0.4 g of SDS, and 10 mg of sodium azide in about 80 mL of deionized water. Adjust to pH 6.8 with 4 *N* HCl, filter, and fill up to 100 mL with deionized water. The buffer can be stored at 4°C for 2 wk.
7. Resolving gel buffer: 1.5 *M* Tris-HCl, pH 8.8, and 0.4% SDS *(9)*. To make 250 mL, dissolve 45.5 g of Trizma base, 1.0 g of SDS, and 25 mg of sodium azide in about 200 mL of deionized water. Adjust to pH 8.8 with 4 *N* HCl, filter, and fill up to 250 mL with deionized water. The buffer can be stored at 4°C up to 2 wk.
8. Acrylamide/*bis*-acrylamide solution: 30% T, 4% C. To make 500 mL of the solution, dissolve 144 g of acrylamide and 6.0 g of *bis*-acrylamide in 300 mL of deionized water. Add 1 g of mixed-bed ion-exchanger resin (e.g., Amberlite MB-1), stir for 10 min, filter and fill up to 500 mL with deionized water. This solution can be stored for 2 wk in a refrigerator.
9. Ammonium persulfate solution: 35% ammonium persulfate. To prepare 1 mL of the solution, dissolve 0.4 g of ammonium persulfate in 1 mL of deionized water. This solution should be prepared fresh just before use.
10. Electrode buffer stock solution (10X): 0.25 *M* Tris-base, 1.92 *M* glycine, 1% w/v SDS. To make 1000 mL of a 10X solution add: 30.3 g of Trizma base, 144 g of glycine, 10.0 g of SDS, and 100 mg of sodium azide. Dissolve in deionized water, filter, and fill up to 1000 mL. Electrode buffer stock solution can be kept at room temperature for 2 wk. Before use, mix 100 mL of the buffer with 900 mL of deionized water.

Table 1
Recipe for Casting Horizontal Pore Gradient Gels (10–17% T, 4% C)

		Resolving gel	
	Stacking gel 6% T	Dense solution 10% T	Light solution 17% T
Glycerol (100%)	3.75 g	2.50 g	—
Stacking gel buffer	2.5 mL	—	—
Resolving gel buffer	—	2.5 mL	2.5 mL
Acrylamide/*bis* soln.	2.0 mL	3.3 mL	5.7 mL
Deionized water	2.5 mL	2.2 mL	1.8 mL
TEMED (100%)	5 µL	5 µL	5 µL
Persulfate (40%)	10 µL	10 µL	10 µL
Final Volume	10 mL	10 mL	10 mL

2.3. Gel Recipes

2.3.1. Gel Recipe for Horizontal Pore Gradient Gels

Add reagents in order given in **Table 1** or **Table 2**.

3. Methods

3.1. Preparation of Horizontal SDS Polyacrylamide Slab Gels

3.1.1. Laboratory-Made Horizontal SDS Pore Gradient Gels on Plastic Backing

1. Assemble the polymerization cassette consisting of two glass plates, one covered with the GelBond PAGfilm, and the other bearing the U-frame (0.5 mm thick) as described in Chapter 22 for casting IPG gels (*see* **Fig. 1A**). Prior to use, wash the glass plates thoroughly with a mild detergent, rinse with deionized water, and let them air-dry. If new glass plates are used, pipet 1–2 mL of repel silane on the glass plate that bears the U-frame and distribute it evenly with a fuzz-free filter paper (KimWipe). Let it dry for a few minutes, rinse again with water, and let it air-dry. Repeat this procedure occasionally in order to prevent the gels from sticking to the glass plates. GelBond PAGfilms should be washed 6 × 10 min with deionized water prior to use to avoid spot streaking upon silver staining *(1)*.

2. Immediately before gel casting, add 5 µL of TEMED and 10 µL of ammonium persulfate to the gel solutions (*see* **Tables 1** and **2**). **Table 2** recipes are ideally suited for running the second dimension of the alkaline-IPGs described in Chapter 22. For casting an SDS gel (size 250 × 195 × 0.5 mm^3) with a stacking gel length of 50 mm, pipet 6.0 mL of stacking gel solution into the precooled (4°C) mold. Then cast the pore gradient on top of the stacking gel by mixing 9.0 mL of the dense (10% T, 25% glycerol) and 9.0 mL of the light (17% T, no glycerol)

Table 2
Recipe for Casting Horizontal Pore Gradient Gels (12–15% T, 4% C)

	Stacking gel 6% T	Resolving gel	
		Dense solution 12% T	Light solution 15% T
Glycerol (100%)	3.75 g	2.50 g	—
Stacking gel buffer	2.5 mL	—	—
Resolving gel buffer	—	2.5 mL	2.5 mL
Acrylamide/*bis* soln.	2.0 mL	4.0 mL	5.0 mL
Deionized water	2.5 mL	1.5 mL	2.5 mL
TEMED (100%)	5 µL	5 µL	5 µL
Persulfate (40%)	10 µL	10 µL	10 µL
Final Volume	10 mL	10 mL	10 mL

solution with the help of a gradient maker similarly as described in Chapter 22 for casting IPG gels. The high glycerol concentration of the stacking gel solution allows overlaying of the pore gradient mixture without an intermediate polymerization step (**Fig. 1B**) (*see* **Note 1**).

3. After pouring, leave the cassette for 15 min at room temperature to allow adequate leveling of the density gradient. Then place it in a heating cabinet at 50°C for 30 min for polymerization. The polymerized gel can be stored in a refrigerator overnight.

3.2. Equilibration of the IPG Gel Strips

The IPG gel strips are equilibrated twice, each time for 15 min in 2 × 10-mL equilibration buffer (*see* **Note 2**). The equilibration buffer contains 6 *M* urea and 30% glycerol in order to diminish electroendosmotic effects *(1)*, which are held responsible for reduced protein transfer from the first to the second dimension. During the second equilibration step, 260 m*M* iodoacetamide are added to the equilibration buffer in order to remove excess DTT (responsible for the "point streaking" in silver-stained patterns) *(10)*. The equilibrated IPG gel strips are slightly blotted to remove excess equilibration buffer and then applied onto the second-dimension SDS gel.

1. Take out the focused IPG gel strips from the freezer, and place them into individual test tubes (200 mm long, 20 mm id). Add equilibration buffer I and 50 µL of the bromophenol blue solution. Seal the test tubes with Parafilm, rock them for 15 min on a shaker (**Fig. 2**), and then pour off the equilibration buffer.
2. Add equilibration buffer II and 50 µL of bromophenol blue solution to the test tube as above, and equilibrate for another 15 min on a rocker.
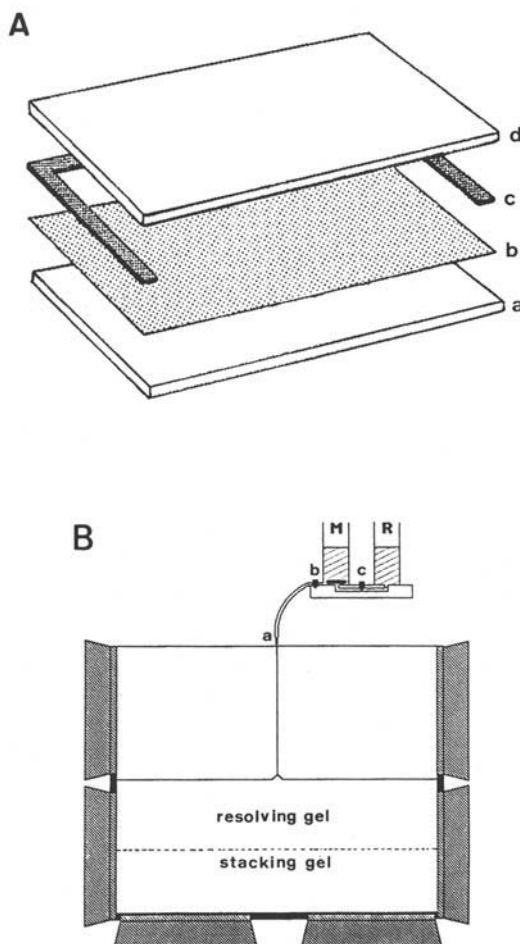
Fig. 1. **(A)** Assembly of the polymerization cassette for the preparation of SDS pore gradient gel cast on plastic backings (a, d, glass plates; b, GelBond PAGfilm; c, U-frame, 0.5 mm thick). **(B)** Casting of SDS pore gradient gel (M, mixing chamber; R, reservoir; a, outlet; b, pinchcock; c, connecting line).

3. After the second equilibration, rinse the IPG gel strip with deionized water for a second, and put it on a piece of filter paper to remove excess equilibration buffer. The strip should be turned up at one edge for a few minutes to help it drain.

3.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the second dimension, laboratory-made or ready-made SDS polyacrylamide gels (0.5 mm thick on GelBond PAGfilm) are placed on the cooling plate of the
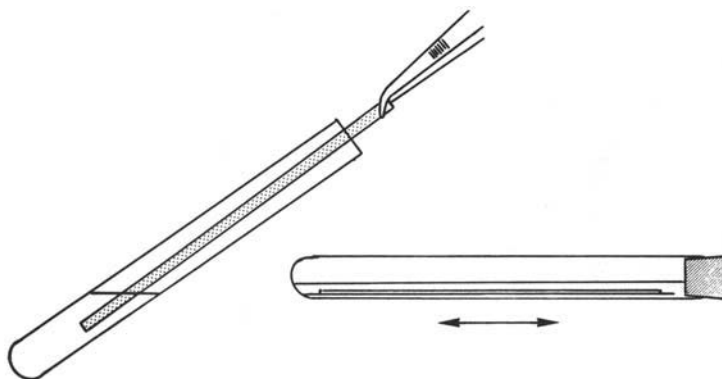
Fig. 2. Equilibration of IPG gel strips prior to SDS-PAGE.

horizontal electrophoresis unit. Electrode wicks (Bio-Rad) or buffer strips from polyacrylamide (Pharmacia) are then applied. The equilibrated IPG gel strip is simply placed gel side down onto the surface of the horizontal SDS gel without any embedding procedure. Horizontal setups are perfectly suited for the use of ready-made gels on film supports. In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels and embedded in agarose (*see* Chapter 26).

3.3.1. Horizontal SDS-PAGE with Laboratory-Made Gels on Plastic Backing

1. Fill the buffer tanks of the electrophoresis unit with electrode buffer. Soak two sheets of filter paper (size 250×195 mm^2) in electrode buffer, and put them on the cooling block (15°C). Soak the electrode wicks (size 250×100 mm^2) in electrode buffer. Place them at the edges of the buffer-soaked filter papers, and perform a prerun (600 V, 30 mA) for 3 h to remove impurities from the electrode wicks. Then remove the filter papers, and discard them. However, the purified electrode wicks remain in the electrode buffer tanks and are used repeatedly.

2. During the equilibration step of the IPG gel strips, open the polymerization cassette, pipet a few milliliters of kerosene on the cooling block (15°C) of the electrophoresis unit, and put the SDS gel (gel-side up) on it. Lay the electrode wicks on the surface of the SDS gel so that they overlap the cathodic and anodic edges of the gel by about 10 mm.

3. Place the blotted IPG gel strip(s) gel-side down onto the SDS gel surface adjacent to the cathodic wick (**Fig. 3A**). No embedding of the IPG gel strip is necessary. If it is desired to coelectrophorese mol-wt (M_r) marker proteins, put a silicone rubber frame onto the SDS gel surface alongside the IPG gel strip, and pipet in 5 μL of M_r marker proteins dissolved in SDS buffer.

4. Put the lid on the electrophoresis unit, and start SDS-PAGE at 200 V for about 70 min with a limit of 20 mA. When the bromophenol blue tracking dye has completely
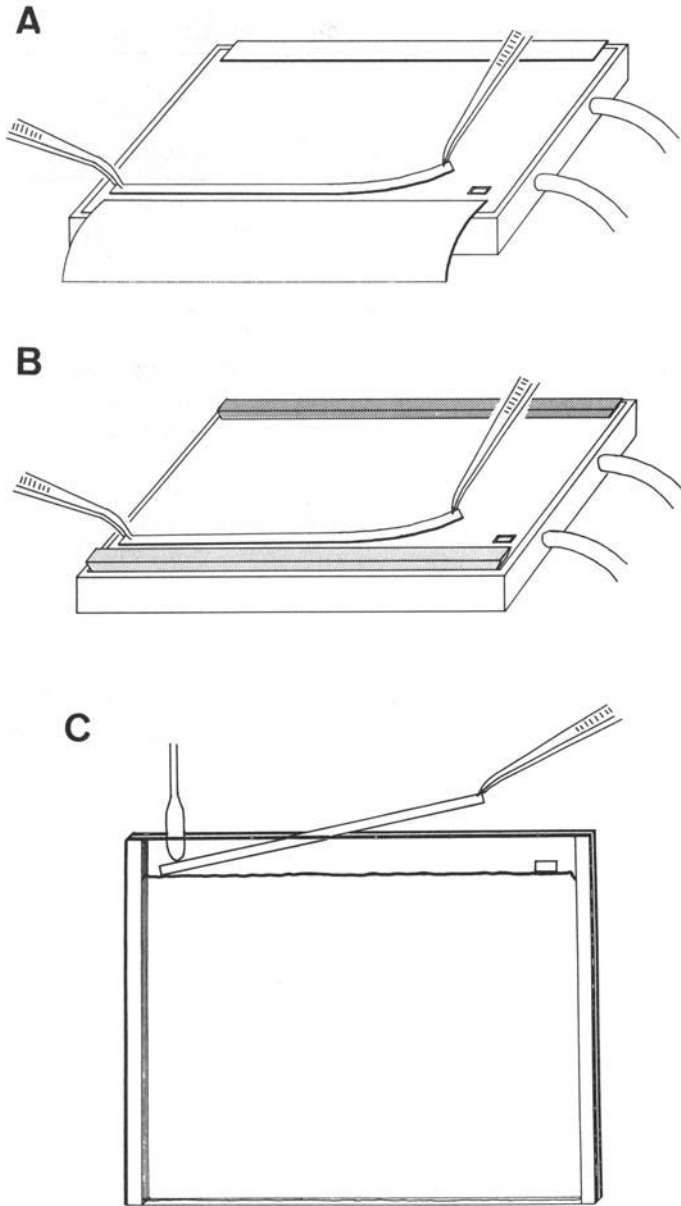
Fig. 3. **(A)** Transfer of equilibrated IPG gel strip onto the surface of a laboratory-made horizontal SDS gel alongside the cathodic electrode wick. **(B)** Transfer of equilibrated IPG gel strip onto the surface of a ready-made horizontal SDS gel along the cathodic buffer strip. **(C)** Transfer of equilibrated IPG gel strip onto the top of a vertical SDS gel.

Table 3
Running Conditions for SDS Pore Gradient Gel (size 250 × 195 × 0.5 mm³)[a]

Time	Voltage	Current	Power	Temperature
75 min	200 V	20 mA	30 W	15°C
5 h	600 V	30 mA	30 W	15°C

[a]Remove the IPG gel strip when the bromophenol blue dye has completely moved out of the IPG gel strip, and move forward the cathodic electrode wick so that it overlaps the former application area.

moved out of the IPG gel strip, interrupt the run, remove the IPG gel strip, and move the cathodic electrode wick forward for 4–5 mm, so that it now overlaps the former sample application area. Put a glass plate (size 200 × 250 mm²) onto the electrode wicks to hold them in place, and continue the run at 600 V with a limit of 30 mA until the tracking dye has migrated into the anodic electrode wick (*see* **Table 3**). Total running time is approx 6 h (running distance: 180 mm). The separated proteins are detected (*see* Chapters 31–38). Alternatively, the gel can be removed from the plastic backing with the help of a film remover (Pharmacia-Biotech) and used for blotting (*see* Chapter 35).

3.3.2. Horizontal SDS-PAGE
on Ready-Made ExcelGel SDS Gradient 12–14

Ready-made SDS gels (ExcelGel SDS Gradient 12–14, size 245 × 180 × 0.5 mm³, on plastic backing) in combination with polyacrylamide buffer strips (Amersham Pharmacia-Biotech) are used.

1. Equilibrate the IPG gel strips as described above (*see* **Subheading 3.2.**).
2. While the strips are being equilibrated, begin the assembly of the SDS ExcelGel for the second dimension: Remove the ExcelGel from its foil package. Pipet 2–3 mL of kerosene on the cooling plate of the horizontal electrophoresis unit (15°C). Remove the protective cover from the top of the ExcelGel and place the gel on the cooling plate, cutoff edge toward the anode. Avoid trapping air bubbles between the gel and the cooling block.
3. Peel back the protective foil of the cathodic SDS buffer strip. Wet your gloves with a few drops of deionized water, and place the buffer strip on the cathodic end of the gel. Avoid trapping air bubbles between gel surface and buffer strip.
4. Repeat this procedure with the anodic buffer strip.
5. Place the equilibrated and slightly blotted IPG gel strip(s) gel-side down on the surface of the ExcelGel, 1 mm apart from the cathodic buffer strip (**Fig. 3B**).
6. Press gently on top of the IPG gel strips with forceps to remove any trapped air bubbles.
7. Align the electrodes with the buffer strips, and lower the electrode holder carefully onto the buffer strips.
8. Start SDS-PAGE at 200 V for about 40 min with a limit of 20 mA. When the bromophenol blue tracking dye has moved 4–5 mm from the IPG gel strip, inter-

Table 4
Running Conditions of ExcelGel SDS Gradient 12–14
(size 245 × 180 × 0.5 mm^3)

Time	Voltage	Current	Power	Temperature
40 min	200 V	20 mA	50 W	15°C
160 min	800 V	40 mA	50 W	15°C

[a]Remove the IPG gel strip when the bromophenol blue tracking dye has completely moved out of the IPG gel strip, and move forward the cathodic buffer strip so that it overlaps the former application area
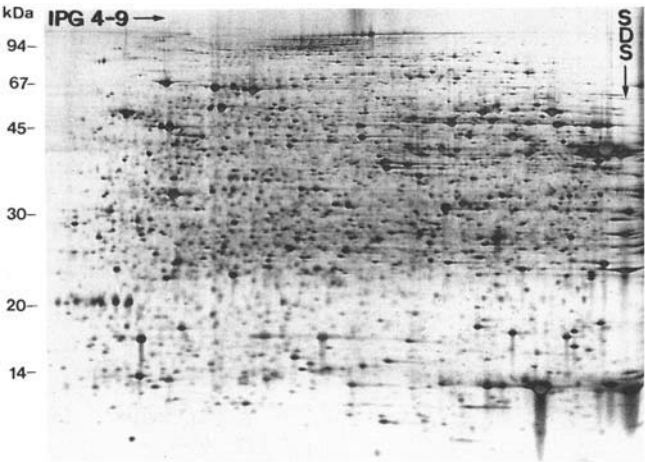

Fig. 4. Horizontal IPG-Dalt of mouse liver proteins. First dimension: IEF with IPG 4-9. Separation distance: 180 mm; sample application: anodic; focusing time: 35000 Vh. Second dimension: horizontal SDS-PAGE, 12–15% T. Silver stain. Reprinted with permission from Görg *(15)*.

rupt the run, remove the IPG gel strip, and move the cathodic buffer strip forward so that it just covers the former contact area of the IPG gel strip. Readjust the electrodes, and continue electrophoresis at 800 V and 40 mA for about 160 min until the bromophenol blue dye front has reached the anodic buffer strip (*see* **Table 4**).

9. Proceed with Coomassie blue or silver staining *(11–14)* (**Fig. 4**) or with blotting (*see* Chapters 31–38).

4. Notes

1. Instead of pore gradient gels, homogeneous SDS gels may be cast for the second dimension.
2. Shorter equilibration times can be applied, however, at the risk that some proteins may not migrate out of the IPG gel strip during sample entry into the SDS-

PAGE. In this case, it is advisable to check, by staining the IPG strip after removal from the SDS gel, whether all proteins have left the IPG strip.

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26

Casting and Running
Vertical Slab-Gel Electrophoresis for 2D-PAGE

Bradley J. Walsh and Benjamin R. Herbert

1. Introduction

The second dimension of 2-D-PAGE uses denaturation and detergent, usually sodium dodecyl sulfate (SDS), to separate proteins by exploiting their molecular mass differences. During SDS-PAGE, all the proteins in the mixture have the same net charge per gram, and movement through the gel is based solely on the molecular mass of the proteins. This is ideal for 2-D-PAGE, because the first-dimension isoelectric focusing is charge-based, and thus, the proteins have been separated by two independent parameters. To achieve a separation based on molecular weight, it is necessary to overcome the intrinsic charges of the protein by loading the protein with the charge modifier SDS, an anionic surfactant with a C12 alkyl tail and an ionic head group. SDS swamps the intrinsic charge of proteins, thus disrupting hydrogen bonds and hydrophobic interactions and preventing protein aggregation. When SDS is used in combination with a reducing agent to cleave disulfide bonds, the proteins are unfolded and form ellipsoid shapes coated in SDS. During electrophoresis in a sieving gel, the denatured SDS polypeptides migrate according to molecular size, and there is a linear relationship between the logarithm of the molecular weight and the relative distance the polypeptides move.

SDS-PAGE is normally performed using a discontinuous buffer system. This type of buffer system causes the proteins in a mixture to be "stacked," in order of mobility, into a series of very fine zones. Because the SDS polypeptides have the same charge-to-mass ratio, they are concentrated into a single fine zone, which is trapped between the leading and trailing ions of the discontinuous buffer. The common buffer system for discontinuous electrophoresis is the

standard Laemmli method *(1)*, which uses glycine as the trailing ion. High glycine levels arising from gel buffer contamination are not desirable when postseparation analyses, such as Edman sequencing or amino acid composition, are required. In this protocol, an alternative buffer system that uses Tricine as the trailing ion is used *(2)*. Another advantage of this buffer system is that the Tricine migrates in front of small polypeptides and resolution of proteins to M_r 5000 is possible.

The pore size of polyacrylamide gels is controlled by varying the amount of acrylamide and crosslinker. The total concentration of acrylamide and crosslinker is referred to as the % T, and is the weight of acrylamide and crosslinker expressed as a percentage of the total volume. The % C is the weight of the crosslinker expressed as a percentage of the total weight of acrylamide and crosslinker. For example, 1 L of a 40% T, 2.5% C stock of acrylamide would have 390 g acrylamide and 10 g of crosslinker.

Second-dimension gels are made as either homogeneous gels, with constant % T and % C, or gradient gels, with increasing % T and usually constant % C. The choice of % T is determined by the molecular weight of the protein that is to be separated. If % T is too low, there will be insufficient sieving, but, if it is too high, the molecules will not penetrate the gel sufficiently.

When separating components in a narrow mol-wt range, homogeneous gels generally give better separation. A suitable % T can be estimated from charts of protein mobility in these gels, but mobility is highly dependent on the buffer used, particularly its pH. Typically a pore gradient gel of 8–18% T is best for crude samples, such as a whole-cell lysates.

Gradient gels have two advantages: they allow proteins with a wide range of molecular weights to be analyzed simultaneously, and the decreasing pore size functions to sharpen the bands, that is, improves resolution. For many applications, the use of a gradient gel gives a superior result. In addition, the portion of the gel where there is a linear relationship between log molecular weight and relative distance is larger in gradient gels (*see* **Note 1**).

2. Materials

2.1. Equipment

1. Protean IIxi multicell (Bio-Rad): A number of commercially available vertical SDS-PAGE units can be used from Pharmacia Biotech (uppsala, Sweden) or ESA (Chelmsford, MA).
2. Protean IIxi multicasting chamber (Bio-Rad): Like the running chambers, other sources for multicasting chambers are available from Pharmacia Biotech or ESA.
3. Gradient former: The Bio-Rad model 385 has a 30- to 100-mL capacity, and the model 395 has a 100- to 750-mL capacity.
4. Stir plate.
5. Power supply.

2.2. Solutions

All solutions should be prepared with Milli-Q water. To avoid protein contamination, it is important to wear gloves when preparing solutions, and handling equipment and samples. Write the date of preparation on all stock solutions. The second-dimension gels should be prepared the day before they are required. Buffer stocks should be stored in the refrigerator and kept for no more than 2 wk.

1. Gel buffer (5X): 0.6 *M* Tris adjusted to pH 6.4 with acetic acid. Dissolve 72.6 g Tris (hydroxymethyl) aminomethane in 800 mL of water and degas for 5 min by sonication. Use glacial acetic acid to adjust the Tris solution to pH 6.4. Make up the volume to 1 L with water.
2. Acrylamide stock: 40% T, 2.5% C. Dissolve 390 g acrylamide and 10 g piperazine diacrylamide in 1 L of water. Add 5 g of Bio-Rad deionizing resin (AG 501 X8 [D], cat. no. 142-6425), and stir for 10 min. If the resin decolorizes, add a further 5 g, and repeat until resin is not decolorized. Filter the solution through Whatman no. 1 paper using a Büchner funnel.
3. Anode electrophoresis tank buffer: 0.045 *M* Tris adjusted to pH 6.6 with acetic acid, 0.1% (w/v) SDS. Dissolve 54.3 g Tris and 10 g of SDS in 9 L water. Adjust to pH 6.6 using glacial acetic acid. Make up the volume to 10 L. The anode buffer should be discarded after three gel runs.
4. Cathode electrophoresis tank buffer: 80 m*M* Tricine, 0.1% (w/v) SDS, adjusted to pH 7.1 with Tris. Dissolve 43.2 g Tricine and 3 g of SDS in 2.5 L water. Adjust to pH 7.1 with Tris. Make up the volume to 3 L. The buffer stock should be made fresh for each gel run.
5. Ammonium persulfate initiator solution: 10% ammonium persulfate. Dissolve 100 mg of ammonium persulfate into an Eppendorf tube, add 900 μL of water, and dissolve with shaking. Prepare fresh each time.
6. Equilibration buffer: 6 *M* urea, 2% SDS, 20% glycerol, 0.12 *M* Tris adjusted to pH 6.4 with acetic acid. Dissolve 14.5 g of Tris, 20 g of SDS and 360 g of urea in 300 mL of water. Use glacial acetic acid to adjust the solution to pH 6.4. Add 200 mL of glycerol, and make up to 1 L with distilled water.
7. Equilibration buffer I: 2% w/v DTT in equilibration buffer. Dissolve 2 g of DTT in 100 mL of equilibration buffer.
8. Equilibration buffer II: 2.5% w/v iodoacetamide in equilibration buffer. Dissolve 2.5 g of iodoacetamide in 100 mL of equilibration buffer.
9. Agarose solution: 1% agarose in anode electrode buffer. Dissolve 1 g of agarose in 100 mL of electrode buffer, and melt in a microwave on medium power.

3. Methods

3.1. Preparing the Multicasting Chamber

1. Prior to assembling the casting chamber, carefully clean the glass plates. Soak the plates in hot laboratory detergent for at least 2 h, and then scrub them with a scouring pad.
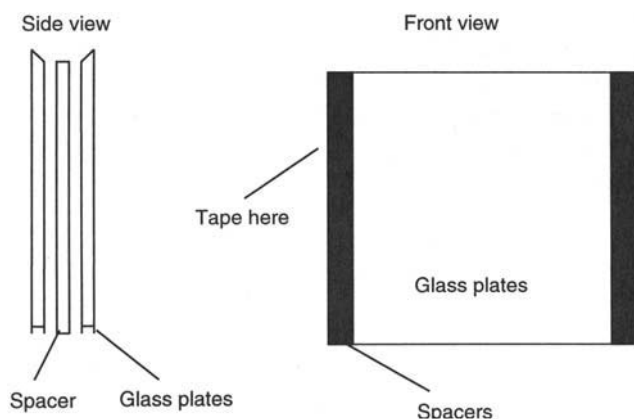
Fig. 1. Method for assembling glass plates.

2. After washing, it is essential that all the detergent is rinsed off with distilled or deionized water. Alternatively, glass plates can be cleaned using a laboratory dishwasher.

3. After rinsing, the plates can be dried and stored in a dust-free environment until required.

4. When assembling the glass plates for casting, wipe each one down with a methanol-soaked tissue and then dry with a lint-free tissue (KimWipes).

5. Place two glass plates together, with the beveled top edges facing in, separated by two spacers positioned along the sides.

6. Tape each set of plates down the sides to ensure the plates and spacers do not move during gel casting (**Fig. 1**). In some systems, for example, the Bio-Rad Protean II, the glass plates are not equal lengths. However, the same basic protocol applies (*see* **Note 2**).

7. When the glass plates have been cleaned and assembled as in **Fig. 1**, place them in the casting chamber following **Fig. 2**. Each set of plates is separated from the next set by a thin plastic sheet. These are usually supplied with a multi casting chamber, but they can be cut from mylar sheets if required (*see* **Note 3**).

3.2. Preparing the Gel Solutions for Casting Gels

1. Before pouring gels, it is important to know the exact volume of the casting chamber when it is full of glass plates (as above). Measure the amount of water required to fill the chamber. For gradient gels, this volume is divided in two. Low- and a high-concentration acrylamide solutions are then made. Thus, when casting six 8–18% T gradient gels in the Bio-Rad Protean II system multicasting chamber (total vol 550 mL), one requires 275 mL of 8% acrylamide solution and 275 mL of 18% acrylamide solution (*see* **Note 4**).
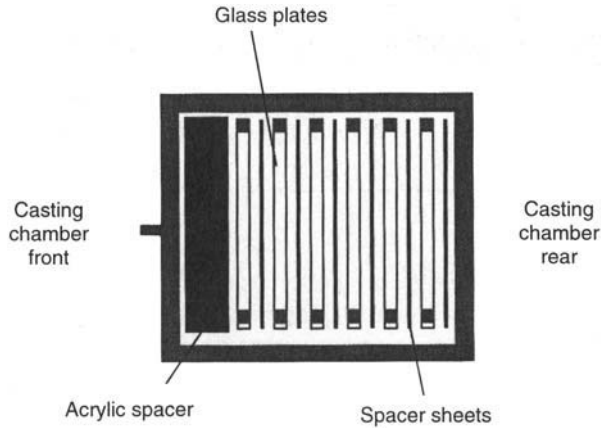
Fig. 2. Method of placing glass plates into the casting chamber of the Protean IIxi Multicell (Bio-Rad Laboratories, Hercules, CA).

Table 1
Formulations for Casting Six 2-mm-Thick Protean II Gels

% T	Buffer mL	Stock acrylamide mL	Milli-Q water mL
8	55	55	165
9	55	62	158
10	55	69	151
11	55	77	144
12	55	83	138
13	55	89	131
14	55	96	124
15	55	103	117
16	55	110	110
17	55	117	103
18	55	124	96

2. Use **Table 1** as a guide for any gradient multicasting system. Once the total volume of the chamber has been established, the volumes of acrylamide stock, buffer stock, and water can be calculated for all the potential gel gradients.
3. Use a measuring cylinder to dispense the appropriate volumes into conical flasks. When preparing the heavy (higher % T) solution, for gradient gels, substitute a 50% (v/v) glycerol in water solution for the water component.
4. Mix the solutions thoroughly, and then remove the dissolved air by degassing. Place the gel solutions into a vacuum desiccator, and evacuate using an oil pump until both solutions have stopped bubbling. This will take between 30 min and 1 h, depending on the volume of solution (*see* **Note 5**).

Table 2
Volumes of Initiators to Add to Each Solution

	Low % T	High % T
TEMED	0.22 μL/mL	0.15 μL/mL
10% APS	2.2 μL/mL	1.5 μL/mL

5. While the solutions are in the desiccator, assemble the gradient maker and peristaltic pump. The gradient maker and peristaltic pump tubing should be rinsed out with Milli-Q water. Turn on the peristaltic pump, and check the flow rate, which should be between 60 and 75 mL/min. After the pump and gradient maker have been assembled and checked, empty the tubing and connect the inlet tubing of the casting unit to the pump tubing. Check that there is a clamp on the inlet tubing of the multicasting unit. This should be opened for gel casting and closed when the casting chamber is full. **Ensure that the clamp is in place and open on the casting unit inlet tubing.**

6. When the solutions, have stopped bubbling, the desiccator should be vented. Prepare the ammonium persulfate initiator solution. Add the initiators to the gel solutions (**Table 2**), and mix by gentle swirling, taking care not to introduce air bubbles (*see* **Note 6**).

7. Pour the gel solutions into the gradient maker. **Check that the gradient maker valves are in the closed position.** Pour the light solution into the mixing chamber and the heavy solution into the reservoir chamber. Start the magnetic stirrer in the mixing chamber, open the outlet valve on the gradient maker, and turn on the pump. Pump the gel solution into the casting chamber at 60–75 mL/minute. Allow the tubing to fill with gel solution, and then open the valve between the mixing and reservoir chambers. When the gels are being poured, it is important to monitor that the magnetic stirrer is mixing correctly.

8. Allow the gel solution to fill the chamber until there is a gap of 0.5 cm at the top of the glass plates. This should require nearly all of the solution in the gradient maker. Do not allow air bubbles to enter the casting chamber. When the solution has filled the casting chamber, switch off the pump, and shut the clamp on the inlet hose of the casting chamber. Remove the pump tubing from the casting chamber inlet, and flush the gradient maker and pump tubing with water.

9. After the gels have been poured, overlay the solution with water-saturated isobutanol to exclude air and ensure a level surface on the top of the gel. Do not move the gels until polymerization has taken place. Allow polymerization to occur for 4–5 h at room temperature, and then replace the isobutanol overlay with 1X gel buffer and store them at 4°C until use. It is important to allow the gels to stand overnight before they are used to enable the polymerization reaction to go to completion. The gels should not be stored for more than a few days before use, since air bubbles will form between the glass plates and the gel.

3.3. Casting Single Percentage Gels

1. Use **Table 1** to make single-percentage solutions when casting homogenous gels. Do not add glycerol to the water used to make up the gels.
2. Add the required amount of initiator, and pour gel solution directly into the casting apparatus. Make sure the clamp on the inlet hose of the casting chamber is shut.
3. Overlay the gels with isobutanol as above, and allow to polymerize.

3.4. Equilibration of IPG Gel Strip

IPG strips must be equilibrated in SDS buffer prior to loading on the second-dimension gel.

1. After IEF or removing from –80°C storage, soak the IPG strip for 10 min in equilibration solution I in a screw-top tube (*see* **Note 7**).
2. Replace solution I with equilibration solution II, and soak the IPG strip for 10 min (*see* **Note 8**).
3. Briefly rinse IPG strips with water, and put on Whatmann paper to remove excess solution.
4. Fill the gap at the top of the gel with the molten agarose solution (*see* **Note 9**).
5. Immediately load the IPG through the agarose, onto the top on the SDS-PAGE gel.
6. Insert the gel cassette into the electrophoresis chamber, and start electrophoresis. It is unnecessary to remove the IPG strip during the electrophoresis run.

3.5. Gel Running Conditions

1. For 2-mm thick gels run for 2 h at 5 mA/gel (*see* **Note 9**), use a buffer-cooling device to keep temperature at 5–10°C. This results in shorter experiment times by allowing higher current to be run through the gel.
2. Increase current to 30 mA/gel until run is complete. The total run time for large gels is approx 16 h (*see* **Note 10**).
3. Allow the bromophenol blue marker dye to migrate to the end of the gels (for vertical slabs).
4. **Gels must be fixed as soon as a run has finished** (*see* **Notes 11** and **12**).

3.6. Fixing Gels

1. On completion of the run, remove slab gels from the apparatus, and open the glass plates.
2. Place gels in 200 mL of 40% methanol, 10% acetic acid in water for 1 h. The bromophenol blue marker will turn a green/yellow color as the fixing solution soaks into the gel (*see* **Note 13**).
3. When gels are to be subjected to autoradiography, fixing should be carried out in 30% isopropyl alcohol, 10% acetic acid for 30 min, since methanol interferes with the fluors.

4. Notes

1. Second-dimension gels can be run horizontally in flat-bed systems or vertically in tank systems. Generally, vertical slab gels are larger and thus offer better

resolution. Additionally, multiple vertical slab gels can be run in large tanks (5–20 gels/tank). A stacking gel is not normally used, especially if the separating gel is a pore gradient.

2. Mini–2-D gels, for example, the mini-Protean II, are very useful for rapid screening of new samples, or evaluating sample preparation techniques. The protocols for running these gels are identical to that for large-format gels. However, the format of most mini-second-dimension systems does not allow for a full-length (11 cm) IPG to be used. Currently, the resolution of mini–2-D-PAGE is not as high as large-format gels can provide. Mid-sized gels, either flat bed (i.e., Excelgel) or vertical slab (Protean II 20 × 16 cm), allow detection of five times the number of spots as mini–2-D systems, and can be used for either analytical or preparative work. Large-format gels (Investigator 22 × 26 cm) resolve more spots, and allow for the inclusion of a number of mol-wt or sample lanes as well as the IPG, but the large size can be difficult to handle, and thicker gels (2 mm) are normally used.

3. SDS-PAGE gels should be poured in a multigel casting chamber to ensure that they are uniform in composition. This is especially important for gradient gels.

4. Small squares of filter paper, which can be used as labels, are placed between the glass plates before the gels are cast. They do not interfere with the running of the gel and are very useful for keeping track of samples when running several gels at a time.

5. After the heavy and light solutions have been prepared, they should be degassed in a vacuum desiccator for 1 h. This will remove any air and prevent oxygen from inhibiting the polymerization. Dissolved gas in the gel solutions may also cause bubbles in the gels during polymerization.

6. When the gel solutions are ready for casting, prepare the ammonium persulfate (APS) initiation solution. It is **very** important to prepare this solution fresh each time gels are polymerized, because old solutions will deteriorate and the rate of polymerization, and hence the pore size, will be different. Add the correct amount of APS and TEMED to each solution, and pour the gels.

7. The urea and glycerol in the equilibration increases the solubility of the proteins and minimize diffusion. DTT is added to reduce and solubilize the proteins.

8. Iodoacetamide is added to remove DTT and alkylate the proteins. The DTT must be removed to minimize vertical streaking in the second dimension.

9. Take care to ensure the agarose solution is hot when it is loaded. If the agarose is too cool, it will set prematurely.

10. To decrease vertical streaking in the second dimension and maximize the amount of protein extracted from the first-dimension, IPG it is useful to run the SDS gels at a low current for the first 2 h. The current can then be stepped up for the remainder of the run.

11. Current settings are dependent on the gel dimensions and thickness, i.e., larger or thicker gels will require more current to run in an equivalent time.

12. Colored markers can be used to monitor the progress of the electrophoresis run. If each gel has a lane of markers, they are easy to overlay for later comparison. Migration distance is determined by molecular weight. The log molecular mass

has a sigmoidal relationship to the distance traveled (expressed as R_f, relative to the moving boundary). The molecular weight for unknown proteins can readily be estimated from a plot using standards of known molecular weight.

13. It is wise to perform a one-dimensional SDS gel experiment (using the same system as your second dimension for 2-DE) with an unknown sample to determine which markers to use. It is sometimes useful to add a known protein to a sample as an internal standard. This can be useful as a control between runs and help to monitor the reproducibility of the 2-DE patterns.

14. The analytical nature of 2-DE makes it more appropriate to use high-sensitivity detection, such as silver staining or autoradiography, rather than the more common staining with Coomassie brilliant blue (CBB). Silver staining can be up to 200 times more sensitive than CBB and permits analysis of dilute samples. As a result, sample overload is much less of a problem. Silver staining may be made up to eight times more sensitive by prior staining with CBB.

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27

Nonreducing 2-D Polyacrylamide Gel Electrophoresis

Hong Ji and Richard J. Simpson

1. Introduction

Several approaches can be used for identifying 2-DE resolved proteins, including microsequencing, immunoblotting with highly specific antibodies, and mass spectrometry-based technologies (for a review of the latter approaches, *see* **ref. *1***). In the case of immunoblotting, although this technique has the advantage of being both fast and specific, it is not always straightforward, since many antibodies fail to recognize proteins following acrylamide gel electrophoresis. This is owing to the fact that some monoclonal antibodies (MAbs) recognize only conformational epitopes, which are dependent on the native spatial conformation of the protein, as distinct from sequential determinants, which are dependent on the linear amino acid sequence of a peptide (for a review, *see* **ref. *2***). In contrast to sequential determinants, conformational determinants are frequently destroyed by reagents employed in electrophoresis (e.g., reducing reagents, SDS, and/or urea) *(3,4)*. Although electrophoretic methods used for resolving native proteins have been described *(5)*, these techniques are not widely used because of technical problems, such as protein aggregation and the wide variance in the effective electrophoretic mobility of proteins in different buffer systems. Moreover, native 2-DE in our hands has proven to be time-consuming compared to conventional reducing 2-DE *(4)*.

This chapter describes a nonreducing 2-DE system in which both first- and second-dimension electrophoresis are performed in the absence of reducing reagent. This method permits the proper refolding of proteins following their electroblotting onto nitrocellulose membrane; it is particularly useful for the immunoblot identification of proteins with disulfide bond-dependent conformational epitopes *(4,6)*, as well as studying protein-protein interactions *(7)*.

2. Materials

1. Multiphor II flat-bed electrophoresis unit (Pharmacia, Uppsala, Sweden).
2. Bio-Rad protein II multicell (Bio-Rad, Hercules, CA).
3. Xcell™ Mini-Cell apparatus (Novex, San Diego, CA).
4. PhosphorImager (Molecular Dynamics, Sunnyvale, CA).
5. Precast gels: Precast isoelectric focusing (IEF) slab gels (pH 3.0–10.0) and 4–20% T gradient acrylamide Tris-glycine gels are from Novex Immobiline Drystrips (pH 3.0–10.0, 18 cm) are from Pharmacia.
6. IEF pH 3.0–10.0 cathode buffer: Novex cat. no. LC5310 (arginine- and lysine-free base).
7. Anode buffer: Novex cat. no. LC5300 (phosphoric acid).
8. Sample buffer: Novex cat. no. LC5311.
9. IPG sample solution with DTT and urea: 9 M urea, 2% v/v Pharmalyte pH 3.0–10.0, 0.5% v/v Triton X-100, 2% w/v DTT, a few grains of bromophenol blue.
10. IPG sample solution without DTT and urea: 2% v/v Pharmalyte pH 3.0–10.0, 0.5% v/v Triton X-100, few grains of bromophenol blue.
11. Rehydration solution with DTT and urea: 8 M urea, 0.5% v/v Triton X-100, 0.5% v/v Pharmalyte, 0.2% DTT.
12. Rehydration solution without DTT and urea: 0.5% v/v Triton X-100, 0.5% v/v Pharmalyte.
13. 12% Acrylamide gel solution: 27.0 mL of 30% acrylamide, 16.9 mL of 1.5 M Tris-HCl, pH 8.8, 0.675 mL of 10% SDS, 22.58 mL of water, 0.34 mL of 10% ammonium persulfate, 23 μL of TEMED. Makes enough for two gels.
14. Equilibration buffer (denaturing, nonreducing): 12.5 mL of 0.5 M Tris-HCl, pH 6.8, 20 mL of 10% SDS, 10 mL of glycerol, 5 mL of water, a few grains of bromophenol blue.
15. Equilibration buffer (denaturing, reducing): 12.5 mL of 0.5 M Tris-HCl, pH 6.8, 20 mL 10% SDS, 10 mL glycerol, 5 mL β-mercaptoethanol, a few grains of bromophenol blue.
16. Laemmli SDS-PAGE running buffer: 0.25 M Tris-base, 1.92 M glycine, 0.1% w/v SDS.
17. Cell-culture medium and fetal calf serum: RPMI 1640 medium is purchased from Irvine Scientific (Santa Ana, CA), and fetal calf serum (FCS) is obtained from Commonwealth Serum Laboratory (Melbourne, Australia). Methionine- and cysteine-free Eagle's minimal essential medium is from ICN Biomedicals (Costa Mesa, CA).
18. Phosphate-buffered saline: 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 and 150 mM NaCl.
19. Trans [^{35}S]-label™ is from ICN Biomedicals.
20. Lysis buffer: 1% Triton X-100, 5 mM Tris-HCl, pH 7.5, 20 μM leupeptin, 10 U/mL trasylol.
21. Gel stain solution: 0.1% w/v Coomassie blue R-250, 50% v/v methanol, 10% v/v acetic acid.
22. Destain solution: 12% v/v methanol, 7% v/v acetic acid.

3. Methods

3.1. Preparation of Total Cellular Lysate

Human colon carcinoma cell line LIM1215 *(8,9)* is cultured in RPMI-1640 supplemented with 10% FCS, 25 U/L insulin, and 1 mg/L hydrocortisone.

1. Plate 10^7 cells in RPMI-1640 medium containing 10% v/v FCS in 150-cm^2 style cell-culture dish, and incubate at 37°C in 5% v/v CO_2 atmosphere for 6 h.
2. Wash cells three times with methionine- and cysteine-free Eagle's minimum essential medium (modified), and culture them in the same medium containing 5% v/v dialyzed FCS and 500 μCi/mL of Trans [^{35}S]-label for 16 h at 37°C.
3. After the cells are radiolabeled, wash them three times with ice-cold PBS, and then add ice-cold lysis buffer (0.5–1.0 mL/10^7 cells) (*see* **Notes 1** and **2**).
4. Incubate the cell lysate at 4°C for 5 min. Vortex the mixture briefly (10–20 s), and then centrifuge (20,000*g*) at 4°C for 10 min to remove insoluble material.

3.2. Non Reducing 2-DE Using Precast IPG Gels *(*see Note 3*)*

1. Rehydrate the IPG gel strips in rehydration solution without urea and DTT for 4–6 h (*see* **Note 4**).
2. Dilute (1:1) the Triton X-100 extracted cellular proteins with IPG sample solution or IEF sample buffer from Novex.
3. Blot the rehydrated strips lightly between two sheets of water-saturated filter paper, and then place them in an Immobiline strip tray (*see* Chapter 21). Position the sample loading wells, and then add the protein samples (maximum volume, 100 μL) (*see* **Notes 5** and **6**).
4. Perform IEF in a flat-bed electrophoresis apparatus at 500 V for 1 h, 1000 V for 1 h, and 3500 V for 40 h at 20°C (*see* **Notes 7** and **8** and Chapter 21).
5. Before performing the 2-D SDS-PAGE, equilibrate the IPG strips for 2 × 20 min in 20 mL nonreducing equilibration buffer.
6. Apply the equilibrated IPG gel strips to 2-D 12% polyacrylamide gels (160 × 160 × 1 mm gel size) and electrophorese the samples at 25°C for 16 h using Laemmli SDS running buffer *(10)* until the tracking dye reaches 1 cm from the bottom of the separation gel.
7. Stain the gels with gel stain solution (~20 min) and then immediately destain them with destain solution (3–6 h, until the spots can be visualized clearly against the background).
8. Analyze the 2-DE gel images of [^{35}S]-methionine and [^{35}S]-cysteine-labeled proteins using a PhosphorImager (*see* Chapter 32).

3.3. Reducing 2-DE Using Precast IPG Gels

Apart from having DTT and urea in the rehydration and the sample solution and DTT in the equilibration buffer, reducing 2-DE is performed as outlined in **Subheading 3.2.**

3.4. Nonreducing 2-DE Using Precast IEF Gels

1. Dilute the Triton X-100 extracted cellular proteins (1:1) with IEF sample buffer (Novex) and load 40 μL of the sample into each well of the precast IEF gel (Novex) (*see* **Notes 9** and **10**).
2. Perform IEF at 25°C in an Xcell™ Mini-Cell apparatus at 100 V for 1 h, 200 V for 1 h, and 500 V for 0.5 h (*see* **Notes 11** and **12**).
3. After IEF, cut the slab IEF gel into gel strips with a gel cutter (*see* **Note 13**).
4. Prior to 2-D SDS-PAGE, equilibrate the IEF gel strips for 20 min in 10 mL of equilibration buffer (denaturing, nonreducing).
5. Apply the equilibrated IEF gel strips to a 2-D 4–20% T precast polyacrylamide gel (80 × 80 × 1.0 mm, Novex).
6. Perform electrophoresis (125 V constant voltage) at 25°C for ~1.5 h using Laemmli SDS running buffer *(10)* until the tracking dye reaches the bottom of the separation gel.
7. After electrophoresis, stain the gels with stain solution for 20 min.
8. Destain the gels with destain solution.
9. Analyze the 2-DE gel images of [^{35}S]-methionine- and [^{35}S]-cysteine-labeled proteins using a PhosphorImager.

3.5. Reducing 2-DE Using Precast IEF Gels

Reducing 2-DE using precast IEF gels is performed as described in the method for nonreducing 2-DE (**Subheading 3.4.**), except that reducing IPG sample solution is used in the 1-D IEF, and DTT is included in the equilibration buffer before performing the 2-D SDS-PAGE.

4. Notes

1. The sample preparation method used in this chapter is a detergent-extraction method. Although the majority of cellular proteins are extracted by 1% Triton X-100, some proteins, such as nuclear proteins and cytoskeleton proteins, may not be fully extracted under these conditions. If an antibody to a particular protein is available, it is always better to run an SDS-PAGE and use this antibody to test whether the protein of interest is in the prepared sample. If the protein of interest is not extracted with 1% Triton X-100, other methods of sample preparation should be evaluated. For a review of extraction methods, *see* **ref. *11***.
2. In commonly used IEF sample solutions, most proteinases will be inactivated because of the presence of 8–9 *M* urea. In contrast, however, many proteinases may be fully active in 1% Triton X-100 extraction solution (without urea). Therefore, it is important that the protein sample preparation step be performed at 0–4°C. Additionally, proteinase inhibitors should be added to the protein extracts. A typical protein inhibitor cocktail may include antipain dihydrochloride, aprotinin, bestatin, chymostatin, E-64, EDTA, leupeptin, pefabloc® SC, pepstatin, and phosphoramidon. Such a protein inhibitor cocktail is now commercially available (Boehinger Mannheim, Castle Hill, NSW, Australia). For various proteinase inhibitor cocktails, *see* **ref. *11***.
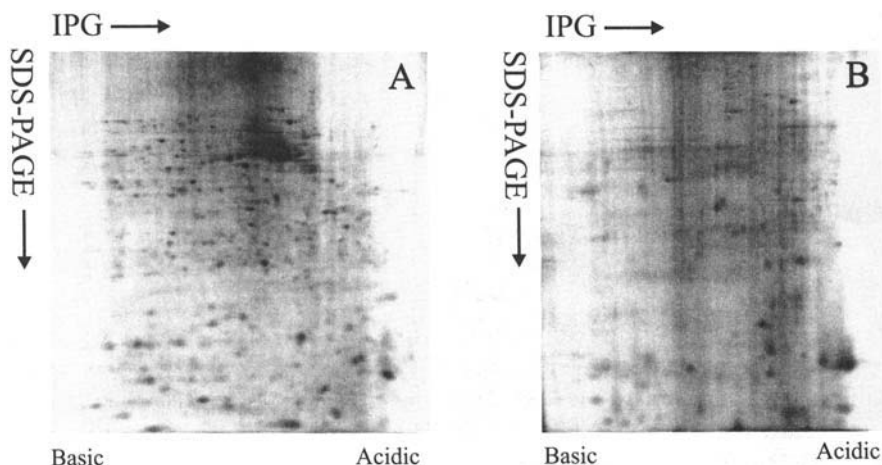
Fig. 1. Distribution patterns of proteins from a Triton X-100 extract of LIM 1215 cells resolved by 2-DE, using IPG as the first-dimension IEF. A detergent extract of LIM 1215 cells was mixed with IPG sample solution, and resolved by 2-DE with (panel **A**) or without (panel **B**) DTT and urea (*see* **Notes 3**, **4**, **14**, and **15**).

3. There are at least three systems that can be used for performing nonreducing 2-DE: precast Novex gel, conventional IEF *(12)*, and IEF with IPG gels *(13–15)*. The major distinction among these systems is the different manner of performing IEF. Precast Novex gels have the advantage of being convenient, time-saving, nonreducing, and nondenaturing, with less sample precipitation in the sample well. However, one disadvantage of the Novex system is that the gel size is relatively small; it is therefore not suitable for resolving large numbers of proteins. Another disadvantage of this system is that prolonged focusing will result in cathodic pH gradient drift. Considering the above-described advantages and disadvantages, the system is suitable for proteins that (a) are well-separated from other adjacent proteins and (b) can be well focused after a short time (approx 2.5 h).

   If immunoblotting is performed after 2-DE, the NOVEX system is certainly a first choice, since using small gels will save reagents, especially antibodies, at the immunoblotting stage. Compared to conventional 2-DE with carrier ampholytes, the usage of IPG has produced significant improvements in 2-DE, in terms of reproducibility and good separation of basic proteins *(13–15)*. IPG gels are routinely used in our laboratory for reducing 2-DE. Using IPG as the first dimension, we have established 2-DE protein databases for human colon carcinoma cells *(16,17)* and for a human breast carcinoma cell line *(18)*. We have also used precast IPG for our nonreducing 2-DE studies. Long focusing times and large gel size have resulted in excellent protein separations. For instance, the isoforms of some high-mol-wt proteins are better separated in this system (**Fig. 1**) compared to that achieved using Novex gels (**Fig. 2**). The major problem with IPG in
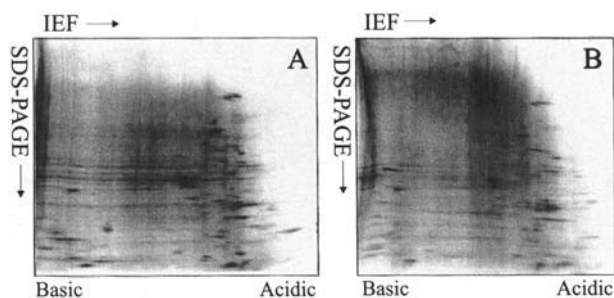
Fig. 2. Protein distribution patterns of a Triton X-100 extract of LIM 1215 resolved by precast 2-DE gels. A detergent extract of LIM 1215 cells was mixed with reducing IPG sample solution and resolved by 2-DE using precast Novex 2-DE gels (panel **A**) or nonreducing IEF sample solution (panel **B**), and resolved by 2-DE using precast Novex 2-DE gels (*see* **Notes 3** and **9**).

nonreducing 2-DE is that without urea in the presence of the gel and sample solution, but many proteins precipitate in the sample loading well. Nonreducing 2-DE with carrier ampholyte can also be performed using the Bio-Rad IEF apparatus, which permits lager gel sizes. This system gives better resolution than small gel systems. Again, the system is not suitable for prolonged focusing and the separation of basic proteins.

4. Nonreducing 2-DE can be performed in the presence or absence of urea. Including urea in IEF, however, increases the solubility of proteins, since it decreases the strength of hydrogen bonds and hydrophobic interactions *(11,19)* (compare panels A and B, **Fig. 1**). This difference is mainly caused by lack of urea in the gel and sample solution. Severe vertical streaks are often observed in the final 2-DE gel patterns if urea is not included (urea facilitates the solubility of protein when they focus at their p*I [11,19]*). If the desired protein spatial conformation can be restored after SDS and/or urea treatment, the inclusion of urea in nonreducing IEF gels is recommended.

5. The loading capacity of sample wells is typically 40 and 100 μL for the Novex gel and IPG gel systems, respectively. When nonreducing 2-DE is performed, protein samples need to be diluted 1:1 with sample solution. For these reasons, protein samples, especially cellular protein samples, should be prepared at high concentrations (e.g., 10^7 cells/0.5 mL).

6. When IPG gel strips are rehydrated in the rehydration solution lacking DTT and urea, the cathodic end of the gel strips swell less compared to rehydration in the presence of DTT and urea. This in turn may lead to the connection between the gel and sample cup being loosened. Therefore, extracare should be paid to the sample cup location step. It is always useful first to load 50 μL of sample buffer to test for possible leaks.

7. Although there is no obvious difference between 2-DE gel patterns obtained by IEF conducted with or without a layer of paraffin oil, we prefer to perform IEF

with the IPG gel strip under a layer of paraffin oil. This is because of the long focusing time required for nonreducing IEF (*see* **Note 8**). For long focusing times, it is recommended that paraffin be used to prevent the evaporation of water.

8. It has been suggested that the optimum focusing time should be 9 h when reducing IEF is performed using IPG gel strips (18 cm long, pH 3.0–10.0) *(20)*. We found that with the same focusing time under nonreducing conditions, the majority of proteins do not focus well. In our experience, with the same gel strips, the focusing time at 3500 V should be 40–48 h for nonreducing IPG without urea.

9. The Triton X-100 extract of colon carcinoma cells is diluted (1:1) with Novex sample buffer and resolved by 2-DE using precast Novex gel. Although the location of some proteins is affected by the presence or absence of reducing reagent, the electrophoretic behavior of a large number of the proteins remained the same, regardless of whether they were focused under reducing (**Fig. 2A**) or nonreducing (**Fig. 2B**) conditions. In general, proteins focus better under reducing conditions.

10. Although the sample wells of Novex gels allow the application of 40 μL of sample, the maximum sample volume should be 35 μL. Smaller sample volumes reduce the chance of crosscontamination of samples when different samples are loaded in adjacent wells.

11. Contamination of anode buffer with cathode buffer causes poor focusing. Both anode and cathode buffer should be applied at least 10 min prior to sample loading. The level of the buffer in the upper reservoir should be checked carefully to ensure that there is no leakage of buffer. If the upper reservoir does leak, the running apparatus should be disassembled and reset.

12. The focusing time for Novex IEF gels recommended by the manufacturer is suitable for most cellular proteins. For some proteins that do not focus well, a time-course should be performed to determine the optimal focusing time. Prolonged focusing time is not recommended, since it will cause the pH gradient to drift toward the cathode.

13. Ten samples can be loaded onto a Novex IEF gel. It is important to cut the gel strips precisely after IEF, especially when protein samples differ and a comparison among the samples is desired. It is useful to lay a piece of ruled paper beneath the gel to guide the cutting.

14. Although the original samples are identical, different 2-DE protein-distribution patterns were obtained. The differences between these two 2-DE patterns may be owing:
    a. Proteins having different p*I* values in native and denaturing gel conditions.
    b. Different p*I*s and the relative molecular mass of proteins in SDS-PAGE varying markedly when disulfide bonds are disrupted (the M_r of protein is usually higher under reducing SDS-PAGE).
    c. The absence of urea in nonreducing IEF causing some proteins to precipitate in the sample loading wells.

15. 2-DE protein distribution patterns of 1% Triton X-100 extracted cellular proteins are similar regardless of whether they are loaded with Novex IEF sample buffer or the sample solution for IPG gels without urea and DTT.

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28

2-D Diagonal Gel Electrophoresis

Joan Goverman

1. Introduction

The task of separating complex mixtures of proteins into individual components was revolutionized by the development of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Modifications of this technique employing gradient gels significantly improved the resolution of proteins and polypeptides based on their molecular weights. Two-dimensional (2-D) gels that combine initial isoelectric focusing in a tube gel with SDS page electrophoresis on a slab gel produce extremely high resolution of proteins. In this method, each protein migrates to a position determined both by its isoelectric point and molecular weight. If the mixture of proteins is complex, however, it can still be difficult to separate all proteins such that they occupy unique positions on the gel, because many proteins have similar isoelectric points and molecular weights.

In situations where the proteins of interest contain interchain disulfide bonds, a different type of 2-D gel can be employed. Diagonal 2-D gels are extremely effective in resolving proteins that contain interchain disulfide bonds from the majority of proteins that do not contain disulfide bonds between chains. Diagonal gels are 2-D gels in which the first dimension is a PAGE run in a tube gel under nonreducing, denaturing conditions. Proteins are thus separated on the basis of their molecular weights before reduction of disulfide bonds. The tube gel is then exposed to a reducing reagent and subsequently transferred to a slab gel for the second dimension. Separation by molecular weight is now performed on proteins following reduction of disulfide bonds. The apparent molecular weights of proteins that do not contain interchain disulfide bonds are the same in both dimensions. Each protein migrates to a position along a
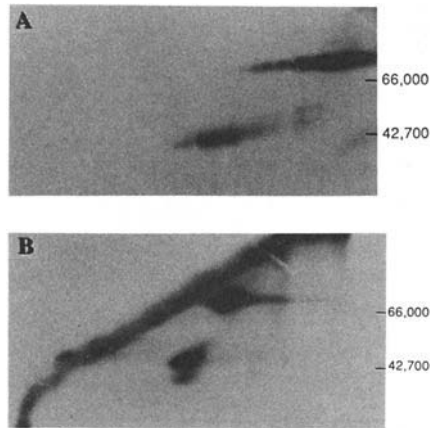
Fig. 1. Comparing the resolution of T-cell receptor polypeptides using two-dimensional gels. **(A)** Isoelectric focusing is employed in the first dimension. **(B)** Nonreducing PAGE is employed in the first dimension. For both gels, the second dimension is standard PAGE on a slab gel. Samples were prepared by labeling cell-surface proteins by iodination and immunoprecipitating proteins from a cell lysate with an antibody specific for the T-cell receptor. Molecular-weight markers are indicated.

diagonal line that is defined by its apparent molecular weight and the length of time of electrophoresis in each dimension. Proteins that are composed of one or more polypeptides connected by disulfide bonds, however, migrate below the diagonal line. In the first dimension, the protein migrates according to an apparent molecular weight that is the sum of its disulfide-bonded polypeptides. Because the protein is reduced before electrophoresis in the second dimension, its components run with correspondingly lower molecular weights. The polypeptides are now separated from the majority of proteins that migrate on the diagonal line formed from non–disulfide-bonded proteins.

Diagonal gel electrophoresis is particularly useful for separating disulfide-bonded polypeptides from non–disulfide-bonded proteins that have similar molecular weights *(1)*. It can also be used to confirm the presence of disulfide-bonded subunits in a protein's structure. The gels shown in **Fig. 1** illustrate the advantages of using diagonal gel electrophoresis for resolution of T-cell receptor proteins. The T-cell receptor is comprised of two polypeptides connected by disulfide bonds. These proteins run as a smear of poorly resolved spots between 30 and 45 kDa on a 1-D gel following reduction of their disulfide bonds. When these proteins are immunoprecipitated with a T-cell receptor-specific antibody, the different components can be resolved well on 2-D isoelectric focusing gels (**Fig. 1A**). Differences in glycosylation of the receptor

polypeptides account for the multiple spots resolved on this 2-D isoelectric focusing gel. Many proteins have similar molecular weights as the T-cell receptor polypeptides, however, and clean immunoprecipitation with little contamination of other proteins is required to visualize these receptor chains clearly using this system. Diagonal gels provide an alternative method of resolving these proteins from other proteins present in cell extracts. This is particularly important if the immunoprecipitation of these proteins is not stringently specific and other proteins are present in the immunoprecipitate. **Figure 1B** illustrates resolution of the same T-cell receptor chains from other cellular proteins using diagonal gel electrophoresis. As in **Fig. 1**, cell-surface proteins were labeled with [125]Iodine and immunoprecipitation using a T-cell receptor-specific antibody carried out on cell lysates. Although other proteins were nonspecifically immunoprecipitated in this experiment, the T-cell receptor proteins are the only disulfide-bonded, labeled proteins migrating off of the diagonal line in the 35- to 50-kDa range. Although the resolution of the different glycosylated forms of the polypeptides is not achieved without isoelectric focusing, this comparison illustrates the power of diagonal gel electrophoresis in resolving disulfide-bonded proteins from other proteins in a complex mixture.

2. Materials

2.1. Reagents

1. 30% Acrylamide/0.8% *bis*-acrylamide stock.
2. 1 *M* Tris-HCl, pH 8.8.
3. 1 *M* Tris-HCl, pH 6.8.
4. 20% SDS.
5. 10% Ammonium persulfate prepared fresh.
6. Siliconizing solution (Sigmacote, Sigma, St. Louis, MO).
7. Acrylamide gel solution: 1.5 mL of 30% acrylamide/0.8% *bis* stock, 1.68 mL of the 1 *M* Tris-HCl, pH 8.8, 2.82 mL dH$_2$O, 50 μL 20% SDS. The final solution is 7.5% acrylamide.
8. 5% Acrylamide stacking gel solution: 460 μL 30% acrylamide/0.8% *bis*-acrylamide stock, 340 μL 1 *M* Tris-HCl, pH 6.8, 2 mL dH$_2$O, 10 μL 20% SDS, 1.4 μL TEMED, 24 μL 10% ammonium persulfate.
9. Sample loading buffer (2X): 4 mL H$_2$O, 4 mL 10% SDS, 1 mL glycerol, 0.15 g Tris base, 20 mg bromphenol blue. The pH of the solution is adjusted to 6.8 with HCl before the addition of the dye and the final volume is brought to 10 mL with distilled H$_2$O.
10. Equilibration buffer: 1.8 mL 1 *M* Tris-HCl, pH 6.8, 3.0 mL glycerol, 3.5 mL 20% SDS, 1.4 g dithiothreitol, 1.7 mL dH$_2$O.
11. Agarose sealing solution: 1 g agarose, 6.25 mL 1 *M* Tris-HCl, pH 6.8, 25 mL 10% SDS, 69 mL dH$_2$O. Heat the solution until the agarose is melted. Divide the solution into 2.5-mL aliquots and stored at 4°C.

2.2. Equipment

1. Tube gel PAGE apparatus, including eight glass tubes 15 cm in length with internal diameter of 2 mm and outer diameter of 3.5 mm.
2. Slab gel PAGE apparatus.
3. Power supply.
4. Vacuum flask.

3. Method

3.1. First-Dimension Electrophoresis

1. Rinse eight glass tubes in siliconizing solution, and dry thoroughly (*see* **Note 1**). Using Parafilm, seal the tubes on one end, and mark the distances of 11 and 11.5 cm from the bottom.
2. Prepare the acrylamide gel solution, and degas by stirring in a vacuum flask under vacuum for a period of 10 min. To initiate polymerization, add 1.4 μL TEMED and 34 μL 10% ammonium persulfate to the acrylamide solution, and mix well.
3. Draw the solution into a 10-cc syringe, and introduce into the tubes using Teflon tubing connected to the end of the syringe. Fill the tubes to the 11-cm mark. Overlay the acrylamide solution in the tubes with 5 μL of distilled H_2O. The polymerization is complete within 20 min.
4. Remove the water from the top of the tube with a gentle flicking motion. Layer the stacking gel solution over the polymerized gel and then overlay with 10 μL of distilled H_2O. The stacking gel polymerizes within 10 min.
5. Prepare the samples containing up to 50 μg protein by adding an equal volume of 2X sample loading buffer *(2)* and boiling for 2 min (*see* **Notes 2** and **3**).
6. Fill the top and bottom reservoirs of the tube gel apparatus with standard Laemmli electrophoresis buffer *(3)*. Load the proteins by underlaying the samples onto the gel (*see* **Note 4**). Carry out electrophoresis at <1 mA/tube until the sample fully enters the stacking gel. The current is then increased to 1–2 mA/tube, and electrophoresis is continued until the dye is close to the bottom of the gel (approx 1.5 h).
7. Remove the gel from the tube by first loosening the bottom of the gel with Laemmli buffer injected between the glass tube and gel with a spinal needle. Loosen the top of the gel in the same manner, and then apply positive pressure to the top of the gel by connecting the gel tube to a 30-cc syringe filled with Laemmli buffer with flexible tubing (*see* **Note 5**).

3.2. Reduction of Proteins

Soak the gel for 30 min in equilibration buffer to reduce the proteins in the tube gels. Equilibration of the gel is carried out in a chemical fume hood (*see* **Note 6**).

3.3. Second-Dimension Electrophoresis

1. Transfer the tube gel to a standard SDS polyacrylamide gel *(3)*. Typically, a 10% slab gel with a 5% stacking gel is used. A somewhat higher or lower percent

acrylamide slab gel may be used, depending on the mol-wt range of the proteins to be separated (*see* **Note 7**).

2. Melt an aliquot of agarose sealing solution in boiling water. In a fume hood, add 125 μL of 2-mercaptoethanol just prior to use.

3. Layer 0.35 mL of the warm agarose solution along the top of the stacking gel. Quickly lay the tube across the top of the slab gel, and pipet 0.65 mL of the agarose sealing solution on top of the tube gel to seal it to the slab gel (*see* **Note 8**). When the agarose has solidified, the gel is ready for electrophoresis (this step must be performed in a fume hood).

4. Carry out electrophoresis of a 10% polyacrylamide slab gel at 5 mA for 14–16 h or at higher voltages for shorter periods of time. Laemmli buffer is used for the electrophoresis. Bromophenol blue (100 μL of a 10 mg/mL solution) is added to the upper reservoir to monitor the extent of electrophoresis. Shortly after electrophoresis is initiated, the dye will form a blue line in the stacking gel and then migrate down the gel. Electrophoresis is completed when the blue line has reached the bottom of the gel (*see* **Note 9**).

5. The proteins may be detected by any of the staining techniques applicable to SDS-PAGE analysis (*see* Chapters 31–38). Proteins of cellular origin may be detected either by metabolically labeling the cells with ^{35}S-methionine or ^{32}P-orthophosphate, or by surface labeling the cellular proteins by iodination with [125]Iodine (as shown in **Fig. 1**). The labeled proteins are then detected by autoradiography.

4. Notes

1. Siliconizing the tube gels prior to use is very important to aid in removing the gels from the tubes after electrophoresis and to prevent formation of air bubbles in tubes of this small diameter.

2. Proteins that will be subjected to electrophoresis can be dissolved in any buffer that does not contain a reducing agent. The addition of sample loading buffer should be sufficient to adjust the final protein solution to a pH of 6.8.

3. Protein standards to be used as mol-wt markers (composed of proteins containing no interchain disulfide bonds) can be added directly to the sample itself prior to boiling. These proteins will fall along the diagonal line according to their molecular weight. Because the standards will fall on the same diagonal line as most of the proteins in the sample, they must be present in excess of the estimated amount of proteins in the sample in order to be distinguishable by Coomassie blue or silver staining. Alternatively, standards can be run only in the second dimension by loading them in a separate well next to the tube gel. This method allows easier identification of the standards, but is not as accurate for determining the molecular weight of proteins that have been electrophoresed in both dimensions.

4. Loading more than 50 μg of protein onto the gel can result in loss of resolution of the polypeptides. Samples should be loaded in a final volume <10–12 μL.

5. It is not important if the stacking gel breaks off of the tube gel during removal of the gel from the tubes.

6. The tube gel must be immersed in the reduction buffer to achieve complete reduction of the proteins. Five milliliters of buffer are sufficient to cover the gel when the tube gel is placed in a reagent reservoir for multichannel pipeters (Costar, Cambridge, MA). To prevent diffusion of smaller proteins following electrophoresis in the first dimension, reduction of proteins should not continue for longer than 45 min.

7. Percentages of acrylamide and *bis*-acrylamide can be varied to optimize resolution of proteins in a higher- or lower-mol-wt range.

8. It is important that the tube gel is sealed to the slab gel with no air bubbles.

9. Reproducibility of diagonal gels is subject to the same parameters that affect standard two-dimensional electrophoresis: consistency in pouring both the tube and slab gels, identical current, and length of time of electrophoresis. In addition, it is important that reduction of proteins always be complete. Increasing the diameter of the tube gel may require additional time for the reduction solution to penetrate the gel completely.

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29

2-D Phosphopeptide Mapping

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1. Introduction

A major mechanism that cells use to regulate protein function is by phosphorylation and/or dephosphorylation of serine, threonine, and tyrosine residues. Phosphopeptide mapping of these phosphorylated residues allows investigation into the positive and negative regulatory roles these sites may play in vivo. In addition, phosphopeptide mapping can uncover the specific phosphorylated residue and, hence, kinase recognition sites, thus helping in the identification of the relevant kinase(s) and/or phosphatase(s).

Two-dimensional (2-D) phosphopeptide mapping can utilize in vivo and in vitro ^{32}P-labeled proteins *(1–6)*. Briefly, ^{32}P-labeled proteins are purified by sodium docyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose filter, and digested by proteases or chemicals. The phosphopeptides are then separated by electrophoresis on thin-layer cellulose (TLC) plate in the first dimension followed by thin-layer chromatography in an organic buffer in the second dimension. The TLC plate is then exposed to autoradiographic (ARG) film or phosphor-imager screen, and the positions of the ^{32}P-containing peptides are thus identified. Specific phosphopeptides can then be excised from the TLC plate and analyzed further by amino acid hydrolysis to identify the specific phosphorylated residue(s) and/or by manual amino-terminal sequencing to obtain the position of the phosphorylated residue(s) relative to the protease cleavage site *(3)*. In addition, mixing in vivo with in vitro ^{32}P-labeled proteins can yield confirmation of the specific phosphorylated residue and the relevant kinase.

2. Materials

2.1. Equipment

1. Multiphor II horizontal electrophoresis apparatus (Pharmacia).
2. Power pack capable of 1000-V constant.
3. Refrigerated circulating water bath.
4. Thin-layer chromatography (TLC) chamber, ~30 L × 10 W × 28 H cm, and internal standard.
5. Speed Vac or lyophilizier.
6. Shaking water bath.
7. SDS-PAGE apparatus.
8. Semidry blotting apparatus (Owl Scientific).
9. Small fan.
10. Rotating wheel or apparatus.

2.2. Reagents

1. Phosphate-free tissue-culture media.
2. Phosphate-free dialyzed fetal bovine serum (FBS). Alternatively, dialyze 100 mL FBS against 4 L dialysis buffer for 12 h, and repeat two more times using 10,000 MWCO dialysis tubing. Dialysis buffer: 32 g NaCl + 0.8 g KCl + 12 g Tris in 4 L, pH to 7.4, with HCl.
3. $^{32}PO_4$-Orthophosphate, 3–5 mCi/tissue-culture dish.
4. Protein extraction buffer (ELB): 20 mM HEPES (pH 7.2), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1–0.5% NP40 or Triton X-100, 1 μg/mL leupeptin, 50 μg/mL PMSF, 1 μg/mL aprotinin, and containing the following phosphatase inhibitors: 0.5 mM NaP$_2$O$_7$, 0.1 mM NaVO$_4$, 5.0 m$_M$ NaF.
5. Rabbit antimouse IgG.
6. Killed *Staphylococcus aureus* cells (Zyzorbin).
7. Protein A agarose.
8. 2X Sample buffer: 100 mM Tris-HCl (6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol.
9. Protein transfer buffer: 20% methanol, 0.037% SDS, 50 mM Tris, 40 mM glycine.
10. 50 mM NH$_4$HCO$_3$, made fresh each usage from powder.
11. 0.5% (w/v) Polyvinyl pyrolidone-360 in 100 mM acetic acid.
12. Sequencing grade trypsin (Boehringer Mannheim 1047 841): Resuspend 100 μg in 1.5 mL fresh 50 mM NH$_4$HCO$_3$, and store 10 μg/150-μL aliquots at –20°C.
13. Performic acid: Mix 1 vol hydrogen peroxide with 9 vol formic acid. Incubate for 1.5 h on ice.
14. Scintillation fluid.
15. TLC cellulose plastic-backed plates, 20 × 20 cm (Baker-flex/VWR).
16. pH 1.9 Electrophorese running buffer: 50 mL 88% formic acid, 56 mL acetic acid, and 1894 mL H$_2$O. Do not adjust pH.
17. Electrophoresis color marker: 5 mg/mL DNP-lysine, 1 mg/mL xylene cyanol FF in 50 μL *n*-butanol, 25 μL pyridine, 25 μL acetic acid, 1.9 mL H$_2$O.

18. TLC chamber buffer: 75 mL *n*-butanol, 50 mL pyridine, 15 mL glacial acetic acid, 60 mL H_2O.

3. Methods

3.1. ^{32}P-Orthophosphate Labeling

1. For in vivo ^{32}P-orthophosphate labeling of cellular proteins, preplate approx 1×10^6 cells in a 10-cm dish. Rinse adherent cells three times with 5 mL phosphate-free media. Suspension cells can be rinsed and collected by centrifugation at ~1800 rpm for 5 min at room temperature or 30°C, aspirate the media, and repeat as above. Add 3–5 mCi of ^{32}P-orthophosphate in 3.5 mL of phosphate-free media containing 10% dialyzed serum to the 10-cm dish and incubate cells at 37°C for 4–6 h (*see* **Note 1**).
2. Aspirate the ^{32}P-containing media with a plastic pipet, and transfer supernatant waste into a 50-mL conical disposable tube. Rinse the cells twice with 10 mL PBS(–) and combine with ^{32}P-media waste in a 50-mL tube, and dispose of properly.
3. Add 1 mL ice-cold extraction buffer (ELB; **ref. 7**) and place dish on a flat bed of ice behind a shield. Tilt dish slightly every 30 s for 3–5 min to cover the cells continually. Collect cellular lysate and debris by tilting dish approx 30° using a P-1000 pipetman tip, transfer to a 1.5-mL Eppendorf tube, and mix. Alternatively, adherent cells can be released by trypsin/EDTA addition, collected, and washed twice in media containing serum to inactivate the trypsin. After the final centrifugation, add 1 mL ELB, mix by using a P-1000 tip, and transfer to an eppendorf tube. Place tube on ice for 20–25 min with occasional mild inverting (*see* **Note 2**).
4. Spin out insoluble particular matter from the cellular lysate in microfuge at 12,000, 4°C, for 10 min. Transfer supernatant to new Eppendorf tube, and preclear lysate by the addition of 50 μL killed *S. aureus* cells, cap tube, and place on rotating wheel at 4°C for 30–60 min.
5. To remove *S. aureus* cells, centrifuge lysate at 12,000, 4°C for 10 min. Transfer lysate to fresh Eppendorf tube, making sure to leave the last 50 μL or so of lysate behind with the pellet. The presence of contaminating *S. aureus* cells in this portion of the sample will reduce the amount of immunocomplexes recovered if included.

3.2. Immunoprecipitation and Transfer of ^{32}P-Labeled Protein

1. Add primary antibody to the precleared lysate supernatant, usually 100–200 μL in the case of hybridoma supernatants and 3–5 μL of commercially purified antibodies. Add 30 μL protein A agarose beads, cap the tube, and place on a wheel at 4°C for 2–4 h to overnight. If primary mouse antibody isotype is IgG1 or unknown, add 1 μL rabbit antimouse IgG to allow indirect binding of the primary antibodies to the protein A agarose *(7)*.
2. After incubation with primary antibody, perform a "1*g* spin" by placing the tube on ice for ~15 min, and aspirate the supernatant to just above the protein A agarose bed level.

Stop aspirating at the top of the agarose beads to avoid drying the beads. This supernatant waste is still highly radioactive. Wash the agarose beads by addition of 1 mL ice-cold ELB, cap, invert tube several times, and centrifuge at 12,000, 4°C, 30 s. Aspirate supernatant, and repeat two to three more times. Again, avoid drying the beads.

3. After the final 30-s spin, aspirate supernatant off of the protein A beads until just dry, and add 30 µL of 2X sample buffer. Boil sample for ~5 min, centrifuge 12K for 10 s, cool tube on ice, and load immunocomplexes onto an SDS-PAGE *(8)*.

4. After running the SDS-PAGE, separate the glass plates, and trim down the gel with a razor blade by removing the stacking gel and any excess on the sides or bottom. Measure the trimmed gel size, and cut six sheets of Whatman 3MM filter paper and one sheet of nitrocellulose (NC) filter to the same size.

5. Soak two sheets of cut 3MM filter paper in transfer buffer, and place on semidry transfer unit. This can be done by filling a Tupperware-like container with transfer buffer and dipping the 3MM paper into it. Place one soaked 3MM sheet on the back of gel and rub slightly to adhere to gel. Then invert the glass plate with the 3MM gel still stuck to it and peel 3MM gel away from the glass with the use of a razor blade. Place it 3MM side down onto the soaked 3MM sheets on the semidry unit. Soak the cut NC filter with transfer buffer, and place it on top of gel followed by three presoaked 3MM sheets on top of filter. Gently squeegee out bubbles and excess buffer with the back of your little finger or by rolling a small pipet over the stack. It is important to squeegee out the bubbles, but avoid excess squeegeeing that would result in drying the stack. Mop up the excess buffer on the sides of the stack present on the transfer unit. Place the top on the transfer unit. In this configuration, the bottom plate is the cathode (negative) and the top the anode (positive).

6. Transfer the ^{32}P-labeled proteins to the NC filter at 10 V constant for 1.5–2 h. The starting current will vary from ~180 to 300 mA, depending on the surface area of the gel and will drop to around 80–140 mAmp by the end of the transfer.

3.3. Trypsinization of Protein on Nitrocellulose Filter

1. Following transfer of ^{32}P-labeled proteins to NC filter, open the transfer unit. Using a pair of filter forceps, place the NC filter, protein side down onto Saran wrap, and cover. Expose the Saran wrap-covered NC filter to ARG film for approx 1–2 h with protein side toward the film. The length of the exposure will vary with the abundance of ^{32}P-content and protein, and with the efficiencies of recovery from immunoprecipitation and transfer. Radioactive or luminescent markers are needed on the filter to determine the orientation, position, and alignment of the NC filter with respect to the ARG film. Using the markers, line up your filter on top of its ARG. This is best done on a light box. Use a razor blade to excise the slice of NC filter that corresponds to the ^{32}P-labeled protein band.

2. Place the NC filter slice into an Eppendorf tube, add ~200 µL of 0.5% PVP-360 in 100 m*M* acetic acid, cap tube, and incubate in a shaking water bath at 37°C for 30 min (*see* **Note 3**).

3. Wash the filter slice five times with 1 mL H_2O and then twice with 1 mL fresh 50 m*M* NH_4HCO_3.

4. Add 10 μg of trypsin in 150 μL of 50 m*M* NH$_4$HCO$_3$ to the NC filter slice. Incubate in a 37°C shaking water bath overnight. Following this incubation, spike the digestion of the NC filter with another 10 μg of trypsin in 150 μL of 50 m*M* NH$_4$HCO$_3$, and incubate at 37°C for an additional 4 h.

5. Vortex the tube containing the NC filter/trypsin sample for 1 min, and centrifuge at 12,000 and 4°C for 30 s. Transfer the supernatant to a new Eppendorf tube. Wash the NC filter slice by addition of 300 μL H$_2$O, vortex for 1 min, centrifuge at 12,000 for 30 s, and combine the supernatants.

6. Freeze the trypsinized ^{32}P-labeled peptide sample on dry ice and then completely dry in a Speed Vac (without heat). This generally takes about 4 h to complete. Prepare the performic acid for the oxidation step (**step 8**).

7. After the sample has been completely dried, add 50 μL of ice-cold performic acid, and place on ice for 1 h. Stop the reaction by addition of 400 μL H$_2$O to the sample, followed by freezing on dry ice and then drying in a Speed Vac.

8. Resuspend the sample in 8–10 μL of H$_2$O. Determine the level of radioactivity by counting 0.5 μL of the sample on a scintillation counter. Usually a total of ≥2000 cpm is sufficient for 2-D phosphopeptide mapping.

3.4. Phosphopeptide Separation: First and Second Dimensions

1. Mark the origin on the TLC plate by lightly touching a pencil to the TLC plate at the position indicated in **Fig. 1**. Apply multiple 0.5-μL aliquots of the trypsinized ^{32}P-labeled peptides to the origin to achieve ≥2000 cpm. Dry the TLC plate thoroughly between each aliquot application by use of a gentle fan. Pay particular attention to adding each subsequent aliquot to the same small area at the origin. Add 0.5 μL of the color marker 3 cm from the top edge of the TLC plate (**Fig. 1**) and then dry the TLC plate under a fan for an additional 30–60 min.

2. Prepare the Multiphor II apparatus for electrophoresis. Place the Multiphor II in a cold room, connect the cooling plate to the cooling circulator bath hoses, and precool to 5°C. Prepare and chill the pH 1.9 running buffer, and add to Multiphor II buffer tanks. Insert the electrode paddles into innermost chambers, and attach the wire connections. We have used the IEF electrodes in direct contact with the cellulose plate; however, using the paddles provided and wicking buffer onto the plate yield the best results (*see* **Figs. 1** and **2**). Place the cooling plate into the Multiphor apparatus. Add 1 L of prechilled pH 1.9 running buffer to each chamber of the Multiphor II. (These instructions are provided with the Multiphor II unit.)

3. Place the loaded TLC plate on top of the cooling plate. To dampen the TLC plate with buffer, first cut a 21 × 21-cm piece of Whattman 3MM paper, and make an approx 1-cm hole at the origin by puncturing the 3MM paper with a pencil. Soak the cut 3MM paper in pH 1.9 running buffer, blotting it between two sheets of dry 3MM paper, and then placing it over the loaded TLC plate sitting on top of the cooling plate. Slowly pipet running buffer onto the 3MM paper until the entire cellulose plate is damp beneath, avoiding excessive puddling. Remove the paper, and wick a single piece of 13 × 21 cm buffer-soaked 3MM filter paper from the buffer chamber onto the 2-cm outer edge on both sides of the plate (*see* **Fig. 2**).
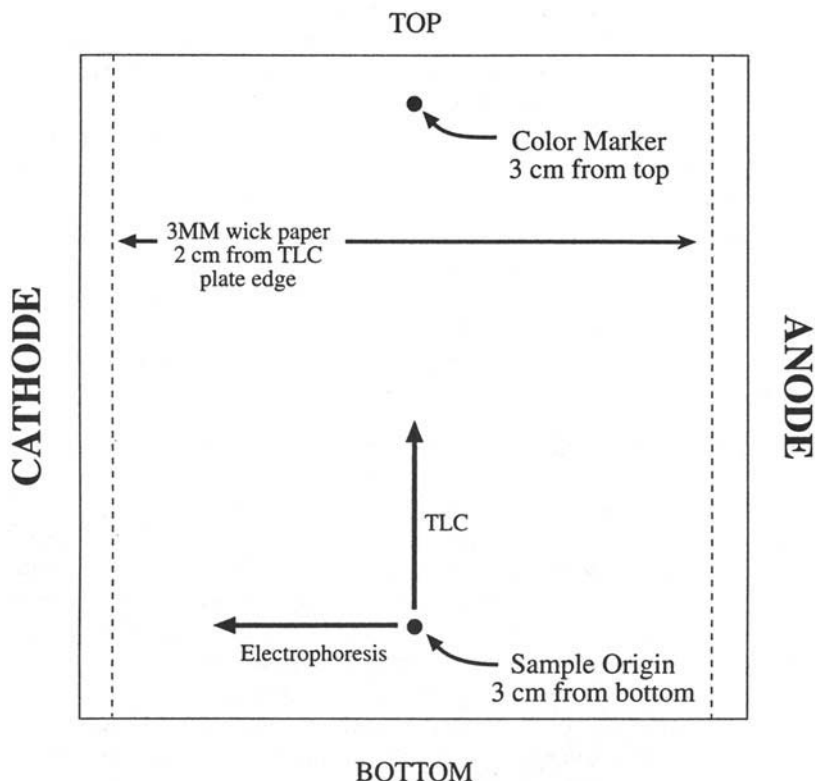
20x20 cm TLC Plate

Fig. 1. The location of the origin, anode, cathode, and color dye marker dye relative to each other on the 20 × 20 cm TLC plate are depicted (*see* **Subheading 3.4.**, **steps 1** and **2**).

Be sure to fold the paper neatly over the edge of the cooling plate, and make sure that it is evenly contacting the TLC plate. Place the glass cover over the TLC plate touching/resting on the 2-cm overhang of the 3MM paper wicks. Attach the Multiphor II cover.

4. Electrophorese the peptides on the loaded TLC plate at 1000-V constant for 28–30 min. The run time may be increased up to 38 min. If further separation of ^{32}P-labeled peptides in this dimension is required, the run time may be increased up to 38 min.

5. Following the first-dimension separation by electrophoresis, remove the TLC plate from the Multiphor II apparatus, and dry for 1 h with a fan.

6. Place the TLC plate on a stand in a thin-layer chromatography chamber pre-equilibrated for 48 h in chromatography buffer. The TLC buffer should cover

Multiphor II Apparatus
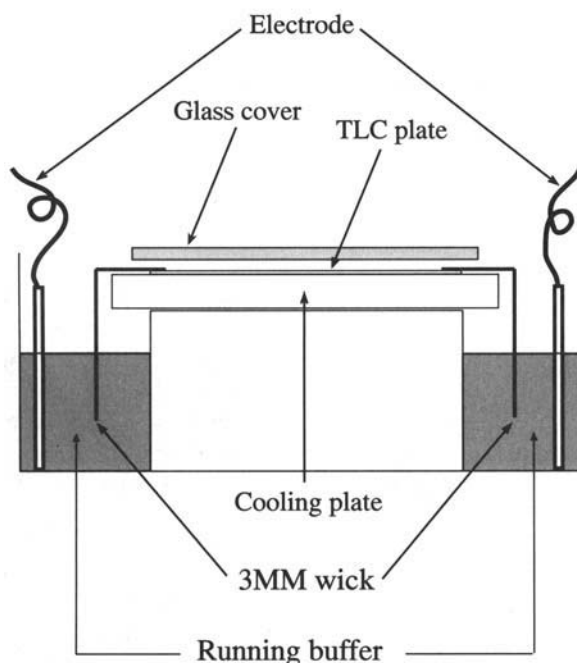
Fig. 2. A cross-sectional view of the Multiphor II apparatus. Loaded TLC plate, Whatman 3MM filter paper wicks, and glass cover plate are depicted in the running position (*see* **Subheading 3.4.**, **steps 2** and **3**).

   approx 1 cm of the bottom of the TLC plate when placed on the stand. Leave the TLC plate in the chamber until the solvent line diffuses to the dye position at the top of the TLC plate, ~3 cm below the top of the plate. This usually takes 7–8 h (*see* **Note 4**).
7. Remove the TLC plate from the chamber and dry for >1 h. Expose the TLC plate to ARG film or a phosphor-imager screen overnight, and develop. If the signal is too weak, expose for 5–7 d. The use of a phosphor-imager greatly diminishes the length of time required to obtain a 2-D phosphopeptide map (*see* **Fig. 3**).

4. Notes

1. We routinely label ~1×10^6 cells; however, depending on the abundance of the specific protein of interest and on the number of phosphorylation sites, this number may vary from 1×10^5 to 1×10^7 cells. In addition, adherent and nonadherent cells may both be labeled in suspension. The cells can be labeled with ^{32}P-ortho-phosphate in a 15- or 50-mL disposable conical tube or T-flask. Please note that
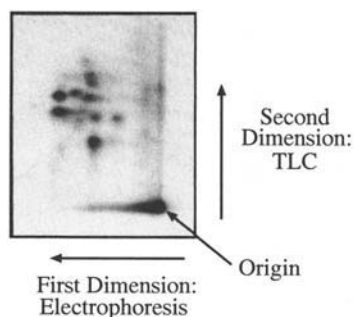
Fig. 3. 2-D phosphopeptide map of the retinoblastoma tumor-suppressor gene product (pRb) labeled with ^{32}P-phosphate in vivo. pRb contains 13 cyclin-dependent kinase (cdk) phosphorylation sites, hence the complexity of the phosphopeptide map. Note the presence of several levels of ^{32}P intensity associated with specific peptides. This can arise by a number of mechanisms, including in vivo site preferences and/or accessibility of the nitrocellulose immobilized ^{32}P-labeled protein to trypsin. The origin, first-, and second-dimension runs are as indicated (*see* **Subheading 3.4.**, **step 7**).

the activity of kinase(s)/phosphatase(s) present in adherent cells may be altered when labeling in suspension. Dish size is not important as long as enough media are added to cover the bottom of the dish/flask. Attempt to achieve a final ^{32}P-orthophosphate concentration of ~1.0–1.3 mCi/mL of media.

2. We use ELB to lyse cells and generate cellular extracts; however, any extraction buffer containing Triton X-100, SDS, DOC, NP40, or similar detergent that will lyse cells will suffice. If a strong background is observed following the SDS-PAGE, transferring the immobilized protein A agarose immunocomplexes to a new microcentrifuge tube prior to the final wash and centrifugation step can result in a reduced background with minimal loss of specific signal.

3. When running multiple lanes of the same ^{32}P-labeled protein immunocomplexes on SDS-PAGE, treat each lane/NC filter slice separately. The trypsinized peptides from as many as five NC filter slices can be combined together, then frozen on dry ice, and dried.

4. Seal the top of the TLC chamber with vacuum grease, and minimize the amount of time the lid is off of the chamber. Pre-equilibrate chamber for >48 h prior to use. We routinely change the TLC chamber buffer every 8 wk. Poor separation in the second dimension is usually indicative of buffer alterations owing to evaporation and/or hydration.

5. The procedures described in this chapter can be stopped at the following steps:
   a. When the immunocomplexes are in 2X sample buffer following the immunoprecipitation.
   b. After lyophilization following trypsinization of the NC filter slices.
   c. After drying the TLC plate following the first-dimension electrophoresis.

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30

Internal Standards for 2-D

Andrew J. Link

1. Introduction

Internal standards are extremely valuable for determining the pH and mol-wt ranges of a specific 2-D gel system and for constructing calibration curves for calculating the relative pI and M_r of unknown proteins. Although external markers are often used for initially calibrating a 2-D gel, eventually the predicted isoelectric points and molecular weights of identified proteins function as calibration markers. For the beginner, several commercial kits are available containing preformulated mixtures of proteins that can be run on 2-D gels either in parallel or mixed with experimental samples as standards. For carrier ampholyte IEF gels, the pH profile can be directly determined by measuring the pH throughout the gel using a standard pH meter. For both CA and IPG IEF gels, a train of differentially carbamylated proteins producing a uniform series of spots on the 2-D gel provides an internal standard for both evaluating the performance of the isoelectric focusing gel and comparing the 2-D spots positions on different gels. However, the exact pI of each spot in the "carbamylated train" is unknown. For calibrating the SDS-PAGE gel, a mixture of protein standards of known molecular weight is simply loaded onto the SDS-PAGE gel flanking both sides of the IEF gel. However, it is important to load the mol-wt standards in the same manner as the IEF gel to minimize any differences in the relative mobilities of the standards and experimental proteins.

2. Materials
2.1. Equipment

1. pH meter.
2. IEF gel tubes.
3. Sample application pieces (Amersham Pharmacia Biotech [Uppsala, Sweden], cat. no. 80-1129-46).

2.2. Reagents

1. 8 *M* urea solution: 8 *M* urea, 0.1% β-mercaptoethanol. Dissolve 2.4 g urea, 0.05 mL of β-mercaptoethanol, and dH_2O in a total volume of 5 mL.
2. 2% Agarose solution: 2% (w/v) agarose. Add 10 mL of dH_2O to 0.2 g agarose and microwave.
3. SDS sample buffer: 10% glycerol, 0.125 *M* Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.016 mg/mL DTT, 0.5% (w/v) bromophenol blue.
4. Mol-wt standards: 0.1 mg/mL of each mol-wt standard dissolved in SDS sample buffer. The choice of which mol-wt standard depends on the mol-wt size range analyzed. The most common markers are rabbit muscle myosin heavy chain (205 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), PMSF-treated bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), β-lactoglobulin (18.4 kDa), sperm whale myoglobin (16.8 kDa), hen egg white lysozyme (14.3 kDa), α-lactalbumin (14.2 kDa), bovine lung aprotinin (6.5 kDa), and bovine insulin chain B (3.5 kDa).

3. Methods
3.1. Determining the pH Profile of a CA-IEF Gel

1. In parallel, run two blank IEF gels without samples along with the normal IEF gels loaded with samples as described in Chapters 15, 16, 17, or 18 in this volume.
2. Add 1 mL of dH_2O to 40–50 glass test tubes (*see* **Note 1**).
3. After focusing the IEF gels, extrude the blank gels and very briefly rinse with dH_2O.
4. Place the blank IEF gel on a glass plate, and cut the gel into 0.5-cm pieces using a sharp single-edge razor blade. A ruler placed under the glass plates can be used as a guide.
5. Place each gel slice into the test tubes containing dH_2O, and place on a shaker for 1 h at room temperature.
6. Using a standard pH meter, read the pH of each solution.
7. Plot the pH profile as function of distance from the top of the gel.

3.2. Preparing Carbamylated Creatine Phosphokinase (CPK) as a p*I* Standard for 2-D Gels

This is adapted from the protocol of Anderson and Hickman *(1)*.

1. Dissolve 5 mg of rabbit muscle CPK in 1 mL of 8 *M* urea solution and aliquot 100 μL into nine 0.5-mL microcentrifuge tubes (*see* **Note 1**). Place one of the aliquots on ice.
2. Put the remaining eight aliquots into a 95°C water bath, and remove one of the tubes at the following time periods: 2, 4, 8, 10, 15, 20, 30, and 45 min and place on ice (*see* **Note 2**).

3. Pool the proteins from the nine tubes, dispense into 50-μL aliquots, and store at –70°C.
4. Run 2 μL (10 μg) of the carbamylated CPK separately and together with your sample during 2-D electrophoresis. The carbamylated CPK should form a train of approx 34 spots across the first dimension, all with approximately the same M_r.

3.3. Mol-Wt Standards for Vertical 2-D Gels

1. Securely cover the bottom of several IEF tubes (1–3 mm id) with Parafilm, and place the tubes upright in a test tube rack.
2. For Coomassie-stained markers, mix 1.5 mL of a set of mol-wt standards with 1 mL of hot, molten 2% agarose. For silver-stained markers, dilute the mol-wt standards solution to 0.01 mg/mL with SDS sample buffer before mixing with the molten agarose.
3. Working quickly, fill the IEF tubes with mol-wt standard solutions using a syringe with a fine needle. Allow the agarose to cool.
4. Carefully extrude the agarose gel from the tubes, and cut into 5-mm pieces.
5. Place each piece into a separate microcentrifuge tube, and store at –70°C.
6. When preparing to run the SDS-PAGE gel, thaw two of the MW tube gel markers and load a marker on each side of the IEF gel (*see* **Note 3**).

3.4. Mol-Wt Standards for Horizontal 2-D Gels (2)

1. After applying the IPG strip to the 2-D flatbed gel (*see* Chapter 25), place one sample application piece at each end of the IPG strip such that the application pieces touch the edge of the IPG gel between the support film of the strip and the SDS gel (*see* **Note 4**).
2. For Coomassie-stained gels, pipet 5 μL of the mol-wt protein standards dissolved in SDS sample buffer onto the application pieces. For silver-stained markers, dilute the mol-wt standards solution to 0.01 mg/mL with SDS sample buffer before loading.
3. Start SDS-PAGE (*see* Chapter 25 for electrical settings and running times).
4. When the bromophenol blue tracking dye has moved out from the IPG gel strip and application pieces, interrupt the run, remove both the IPG gel strip and application pieces, and move the cathodic buffer wick or strip forward as described in Chapter 25. Readjust the electrodes, and continue electrophoresis until the bromophenol blue dye front has reached the anodic buffer strip (*see* Chapter 25 for electrical settings and running times).

3.5. Mixing Internal Standards Directly with the Sample

To minimize differences in the migration of internal standards and experimental proteins, internal standards may be mixed with sample proteins to calibrate your 2-D gel system. However, the standards may interfere with the migration and identification of unknown proteins. Therefore, the standards are generally run in parallel with the biological samples on a separate reference gel. Sets of commercially available protein standards that can be run as either inter-

Table 1
Commercially Available p*I* and mol-wt Standards for 2-D Gels[a]

Protein	p*I*	Mol wt, kDa
Hen egg white conalbumin	6.0, 6.3, 6.6	76.0
Bovine serum albumin	5.4, 5.5, 5.6	66.2
Bovine muscle actin	5.0, 5.1	43.0
Rabbit muscle GAPDH	8.3, 8.5	36.0
Bovine carbonic anhydrase	5.9, 6.0	31.0
Soybean trypsin inhibitor	4.5	21.5
Equine myoglobin	7.0	17.5

[a]Bio-Rad's 2-D SDS-PAGE standards (Bio-Rad, Hercules, CA, cat. no. 161-0320).

nal or external p*I* and mol-wt markers are available from Bio-Rad (Hercules, CA), Sigma (St. Louis, MO), and Amersham Pharmacia Biotech. **Table 1** lists the p*I* and mol-wt ranges of Bio-Rad's 2-D standards.

4. Notes

1. In addition to CPK, carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase can be used. Carbamylated creatine phosphokinase, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, and myoglobin, are commercially available as p*I* markers from Amersham Pharmacia Biotech (Uppsala, Sweden) and Sigma Chemical (St. Louis, MO).
2. During heating, the cyanate generated from urea causes the carbamylation of CPK. The process is adding a carbamyl group (NH_2CO^-) to amino groups on the protein, and the loss of free amino group results in a unit change of the charge of CPK. The products of differentially carbamylating CPK appears as a row of spots with approximately constant M_r and is referred to as a "carbamylation train" *(1)*.
3. Load the markers in the same manner as the IEF gel to minimize differences in the mobility of the standards and the sample proteins. Using the distance migrated by the standards from the top of the gel, a plot of $\log_{10}$ molecular mass of the standards vs the distance migrated is used to estimate the relative mobility (M_r) of unknown 2-D proteins. However, this relationship is only linear over a portion of the gel depending on the gel concentration.
4. The Amersham Pharmacia Biotech IEF sample application piece can be cut in half and the two pieces used for one gel.

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31

Autoradiography of 2-D Gels

Andrew J. Link

1. Introduction

Autoradiography is used to visualize and quantitate radiolabeled proteins that are resolved by 2-D protein gel electrophoresis. Proteins are commonly labeled in vivo with either ^{3}H, ^{14}C, ^{35}S (low-energy β-emitters), ^{32}P (high-energy β-emitter), or ^{125}I (high-energy γ-rays). During film-based autoradiography, these emitted particles or γ-rays enter the film and cause the ejection of electrons from silver halide crystals generating local precipitates of silver atoms. In the past several years, the use of phosphor imaging has been replacing film-based autoradiography. Phosphor imaging has 10–250 times increased sensitivity compared to film-base autoradiography. While film has a dynamic range of about 300 to 1, phosphor imaging has a linear dynamic range over several orders of magnitude *(1)*.

Organic scintillants are used to obtain enhanced autoradiograms of the weak β-emitting isotopes ^{3}H, ^{14}C, ^{35}S *(2,3)*. In fluorography, the scintillants fluoresces on absorption of the β-particle and produces a light photon that forms an image on the film. Because the low-energy β-particle produced from ^{3}H decay cannot penetrate the gel and expose the film directly, fluorography is essential for detecting proteins labeled with ^{3}H. For ^{14}C and ^{35}S labeled proteins, fluorography enhances the detection by approx 15-fold compared to direct autoradiography *(5)*. For strictly high-energy ^{32}P- and ^{125}I-isotopes, an intensifying screen can be used to enhance the film image *(4)*. The intensifying screen absorbs the emission of these isotopes that frequently pass through the film and fluoresces, producing light protons. Although intensifying screens will enhance the film image by fourfold or more, loss of image resolution will occur owing to the light scatter.

This chapter provides protocols for using autoradiography and fluorography to detect radiolabeled proteins after 2-D gel electrophoresis.

2. Materials

2.1. Equipment

1. X-ray film.
2. Saran wrap.
3. Whatman 3MM paper.
4. Gel dryer with a vacuum pump and trap.
5. Photographic flash or stroboscope with an orange filter (Kodak Wratten no. 22A).
6. Whatman no. 1 filter paper.
7. X-ray film holder.
8. Aluminum foil.

2.2. Reagents

1. PPO/DMSO solution: Dissolve 220 g of 2,5-diphenyloxazole (PPO) in 800 mL of dimethyl sulfoxide (DMSO). Store at room temperature.
2. Fix solution: 10% acetic acid, 20% methanol, 3% glycerol.

3. Methods

3.1. Fluorography of 2-D Gels

Fluorography is required for autoradiography of ^{3}H-labeled 2-D proteins and increases the sensitivity of detection for ^{14}C- and ^{35}S-labeled 2-D proteins. It is generally not used for 2-D proteins labeled with ^{32}P or ^{125}I. The proteins in the 2-D gel can be stained and the image recorded as described in Chapters 33 and 34 prior to fluorography, but the staining will decrease the intensity of the film image. Commercially fluorography reagents, such as En^{3}hance from New England Nuclear, are available, but have a reputation of being less sensitive than PPO/DMSO for fluorography *(5)*.

1. Carefully remove the 2-D gel from the glass plates, and place the gel in a glass dish filled with DMSO (at least 10× the volume of gel). Soak the gel for 20 min with gentle agitation.
2. Carefully replace the DMSO, with the same volume of fresh DMSO and soak the gel for 20 min.
3. Replace the DMSO with PPO/DMSO solution. Soak the gel with gentle agitation for 1 h.
4. Replace the PPO/DMSO solution with dH$_2$O. Wash the gel for 20 min with gentle agitation. Since it is insoluble in H$_2$O, PPO will precipitate in the gel matrix and turn the gel opaque.
5. Repeat washing the gel at least twice for 20 min with dH$_2$O or overnight.
6. Dry the gel as described in **Subheading 3.2.**

3.2. Drying 2-D Gels for Autoradiography

Before drying, the 2-D gel can be silver or Coomassie stained as described in Chapters 33 and 34 and the image recorded. After autoradiography, the film can then be compared to the stained 2-D protein pattern. As an alternative to drying the gel, the 2-D proteins can be electrotransferred to a membrane and stained as described in Chapters 35 and 36. The dried membrane can then be used for autoradiography.

1. Carefully remove the 2-D gel from the glass plates and place in a dish filled with at least 10x vol of fix solution. Soak the gel for 1 h (*see* **Note 1**).
2. Place the gel on a piece of Saran wrap slightly larger than the gel.
3. Place a sheet of dry Whatman 3MM paper on the gel (*see* **Note 2**). Try to avoid trapping air bubbles, but do not attempt to remove the 3MM paper once contact has been made with the gel.
4. Place a second sheet of Whatman 3MM paper on a gel dryer.
5. Arrange the sandwich of 3MM paper/gel/Saran wrap on the drying surface of the gel dryer. The Saran wrap must be the top layer.
6. Cover with the sheet of silicon rubber, close the lid of the gel dryer, and apply vacuum suction. Check for a tight seal around the gel. After 20 min, apply heat (60°C) to speed up the drying (*see* **Note 3**).
7. Continue until the gel is completely dry (*see* **Note 4**).
8. Remove the gel that is now attached to the Whatman paper from the gel dryer. Discard the Saran wrap as radioactively contaminated waste and proceed with autoradiography (**Subheading 3.4.**).

3.3. Pre-Exposing X-ray Film Prior to Autoradiography

Since film has a nonlinear dynamic range, the autoradiographic image of a 2-D gel will not be proportional to the amount of radioactivity in the 2-D spots unless the film is pre-exposed to a hypersensitizing flash of light (*see* **Note 5**).

1. Cover the flash or stroboscope with the orange filter.
2. While in total darkness, cover the film with a piece of Whatman no. 1 filter (*see* **Note 6**).
3. Place the covered film perpendicularly at least 50 cm from the light source and expose a series of test films to the light source for different lengths of time (≤ 1 ms) (*see* **Note 7**).
4. Develop the test film.
5. Cut the exposed film into pieces and measure the absorbance of the different exposure times at 540 nm using a spectrophotometer against a blank consisting of film that was not exposed. Choose the exposure time that causes the absorbance to increase by 0.15.
6. Pre-expose the X-ray film using the optimal exposure time and distance. Proceed with autoradiography of the dried 2-D gel.

3.4. Autoradiography of the Dried 2-D Gel
or Electrotransferred Membrane

1. In a darkroom, place the dried gel or membrane in a light-tight X-ray film holder and cover with a sheet of X-ray film (*see* **Note 8**). An alternative to film-based autoradiography is using storage phosphor imaging as described in Chapter 32 of this volume. However, phosphor imaging is not compatible with fluorography.
2. Expose the film for several different lengths of time (*see* **Note 9**). If fluorography or an intensifying screen is used, the X-ray film must be exposed at –70°C (*see* **Note 10**).
3. In a darkroom, quickly remove the film and develop immediately.

3.5. Dual Isotope Detection Using X-ray Film

For double-label experiments using 2-D gels, the following X-ray film-based protocols can be used for the differential detection of $^3H/^{14}C$- and $^{35}S/^{32}P$-labeled proteins. The methods are adapted from the published protocols of McConkey *(6)* and Cooper and Burgess *(7)*.

1. For 2-D gels, run with a mixture of differently labeled $^3H/^{14}C$-proteins, prepare the gel for fluorography as described in **Subheading 3.1.** and dry the gel as described in **Subheading 3.2.**
2. For the detection of both ^3H- and ^{14}C-proteins, perform autoradiography using "screen-type" X-ray film at –70°C as described in **Subheading 3.4.**
3. For the detection of ^{14}C-labeled proteins only, perform autoradiography using a "no- screen" X-ray film at –70°C (*see* **Note 11**).
4. For 2-D gels run with a mixture of differentially labeled $^{35}S/^{32}P$-proteins, dry the gel as described in **Subheading 3.2.**
5. In total darkness, arrange the dried gel in the hybridization cassette in the following order: X-ray film, dried gel (filter paper backing facing the aluminum foil), aluminum foil (15 μm), X-ray film, intensify screen (*see* **Note 12**).
6. Expose the film at –70°C and develop.

4. Notes

1. When drying 2-D gels, cracking of the gel is a constant concern, especially for thick (>1.5 mm) or high %T (>15%) gels. An alternative to using the fix solution with glycerol is to soak the gel in a solution of 2% DMSO, 10% acetic acid in water for 1 h before proceeding with drying. However, using DMSO will increase the time it takes for the gel to dry. If the 2-D gel has been fixed in a high-percentage methanol fix solution (e.g., 50% methanol, 10% acetic acid), but not stained, the gel should be soaked in the 20% methanol fix solution to swell the gel back to its original size.
2. The Whatman 3MM paper should be cut 1–2 in. larger than the gel, but large enough to fit in the gel dryer. If the gel needs to be optically scanned after drying, sandwich the gel between two pieces of porous cellophane to avoid the gel adhering to the 3MM paper during drying. Avoid trapping air bubbles between the gel and cellophane.

3. For a thick gel, a lower temperature during drying (40°C) may be used to prevent the gel from cracking, but will increase the time it takes for the gel to dry.

4. To avoid cracking the gel, it is extremely important that the gel be completely dry before removing it from the gel dryer. When heat is applied, the drying time is ≈1–2 h for a 0.5- to 1.0-mm-thick gel. The drying time will increase with increased %T, gel thickness, and surface area of the gel. However, excessive drying should be avoided to prevent the gels from becoming excessively brittle and fracturing.

5. An alternative to pre-exposing the X-ray film is to include a calibration gel strip that contains known levels of radioactive protein *(8)*.

6. The porous paper acts as a diffuser to avoid uneven fog levels on the film.

7. The film should be at least 50 cm away from the light source to prevent uneven illumination.

8. The type of film used for autoradiography of the 2-D gel depends on whether or not fluorography or an intensifying screen is used. When these image enhancers are used, a "screen-type" film must be used, such as Kodak X-OMAT AR or Fuji RX.

9. Owing to the limited dynamic range of X-ray film, different exposure times allow visualization and quantification of a range of proteins with different abundance. Spots that exceed an absorbance of 1.4 (A_{540}) have saturated the available silver halide crystals and cannot be accurately quantified. Depending on the radioisotope used, the image enhancers employed, and the abundance of the protein, the exposure times can range from hours to several weeks.

10. The exposure at low temperature stabilizes the silver atoms and ions that will form the image.

11. The fluorographic X-ray film of the gel shows the proteins labeled with either or both the ^{3}H- and ^{14}C-isotopes. However, the no-screen film, which is insensitive to light, only detects the ^{14}C-labeled proteins by direct autoradiography, since the ^{3}H decay cannot penetrate the gel and expose the film directly. An optimal ratio of ^{3}H to ^{14}C counts is (8:1), so that the sensitivity of detection of ^{3}H will be equal to the ^{14}C detection by fluorography *(6)*.

12. The first X-ray film directly detects the ^{35}S-labeled proteins. However, the filter paper and the aluminum foil shield the second X-ray film, and only the high-energy ^{32}P-emission is recorded. An optimal ratio of ^{35}S to ^{32}P counts is (50:1) *(7)*.

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32

Double-Label Analysis

Kelvin H. Lee and Michael G. Harrington

1. Introduction

The detection of radiolabeled proteins is of fundamental importance in experimental biology. ^{35}S- and ^{14}C-labeled proteins can provide investigators with information about protein expression, synthesis, and degradation. Moreover, posttranslational modifications, such as phosphorylation, can be studied with the use of ^{32}P. Traditional methods for detecting radiolabeled proteins involve somewhat lengthy exposures to photographic film. Quantitation from film-based autoradiography can be achieved by densitometry of repeated and various exposures to films because of the small linear dynamic range of photographic film.

Storage phosphor screen imaging technology has several advantages over film-based autoradiography for the detection of radiolabeled proteins. These advantages include a nearly linear dynamic range, which covers five orders of magnitude, fast imaging times, and no requirement for wet chemicals. The dynamic range of phosphor screens offers greater sensitivity than film, as well as simple and accurate quantitation of radioactivity. Moreover, the ability to image radioisotopes without using photochemicals results in a more environmentally friendly system that does not require darkroom facilities and has a lower cost per sample exposure. Among the few drawbacks to phosphor imaging technology are the initial cost of the instrument and screens, and the inability to measure all isotopes with a single screen.

One of the most important developments from the use of storage phosphor technology has been in the imaging of samples containing two different radiolabels. In double-label experiments, different biochemical events are tracked simultaneously in the same sample using different radioisotopes. For example,
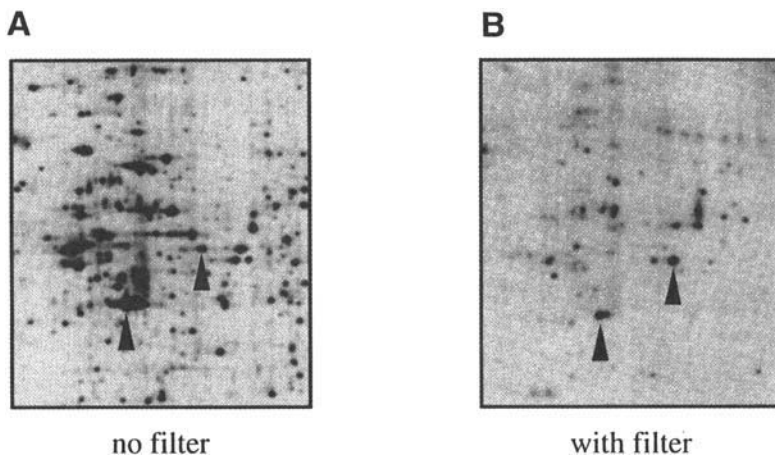
Fig. 1. **(A)** and **(B)** depict a region of a 2-D gel of total protein extract from PC12 cells. These cells were labeled with both ^{35}S-methionine and ^{32}P-orthophosphate. In A, the dried 2-D gel was exposed to a storage phosphor screen for 2 h. In B, the same 2D gel was used to image ^{32}P by placing a copper filter between the gel and the imaging screen. This exposure was for 20 h. Two reference proteins are designated with arrows.

protein expression can be monitored by ^{35}S, whereas a cellular response to a particular stimulus (e.g., protein phosphorylation) can be tracked with ^{32}P. In concert with two-dimensional (2-D) electrophoresis of proteins, double-label experiments can provide a wealth of information on the proteome in a single experiment.

The ongoing concern about the reproducibility of 2-D gels has often limited their use in quantitative and complicated experiments. Storage phosphor screen imaging technology enables users to image a single 2-D gel that has dual-labeled proteins in a rapid manner. The generation of 2-D gel images that detail information about different biochemical events derived from a single 2-D gel bypasses concerns about reproducibility. That is, the resulting images precisely align because they are generated from the same gel.

An example of this approach is shown in **Figs. 1A** and **1B**. **Figure 1A** depicts a region of a 2-D gel of total protein extract from PC12 cells grown on DME media and supplemented with nerve growth factor as described elsewhere *(1)*. These cells were labeled with both ^{35}S-methionine for total protein and with ^{32}P-orthophosphate for phosphoproteins in media that were depleted of methionine and sodium phosphate. ^{35}S was incorporated for 2 h in 2 mL of methionine and phosphate-deficient media containing 250 μCi/mL of ^{35}S-methionine (SA 1000Ci/mmol) and 250 μCi/mL ^{32}P-orthophosphate (370 Bq/mL); 4.5×10^6 cpm of ^{35}S and 4.5×10^4 cpm of ^{32}P were applied to each 2-D gel (corresponding to roughly 3×10^5 cells). In **Fig. 1A**, the phosphor screen was exposed to the

doubly labeled proteins for 2 h, and, in **Fig. 1B**, the phosphor screen was exposed in the presence of a copper filter to the proteins for 20 h. This copper filter acts to attenuate the ^{35}S signal resulting in the imaging of only those proteins containing ^{32}P.

Because these two images were derived from a single 2-D gel, they can be superimposed precisely. As a result, spots that appear in **Fig. 1B** represent phosphoproteins. Spots that appear in **Fig. 1A**, but whose intensity is significantly attenuated in **Fig. 1B**, represent proteins that are not phosphorylated. Using this approach, global protein phosphorylation in response to particular stimuli or growth conditions can be determined by a second 2-D gel of protein extract from this different metabolic state. Such a comparison will yield information not only about those proteins that are phosphorylated in certain situations, but also about the subset of proteins that are dephosphorylated in those same situations. Since the 2-D patterns of ^{32}P-orthophosphate- and ^{35}S-methionine-labeled proteins are grossly different, it is not possible to match these accurately on two separate 2-D samples. Thus, the use of storage phosphor imaging and double-label analysis can yield a great deal of information about the proteome.

Here we present our methods that describe the use of double-label imaging techniques to identify phosphoproteins from cultured cells. In the future, this approach can be extended to the study of other posttranslational modifications given a sufficient filtering distinction between isotopes used to identify various posttranslational modifications. It is important to mention that most of the advantages of double labeling with a radioisotope might be realized in the future with multiple fluorescent markers, as is currently implemented in many of the current automated DNA sequencers. However, this approach requires both instrumentation and fluorescence labeling chemistry to be developed to the same level as exists today for radioactivity.

2. Materials

There are several phosphorimaging devices and screens that are currently available. Because each instrument and screen has slightly different characteristics, some comments on the equipment needed for performing double-label experiments are provided below.

2.1. Equipment

1. Phosphor imager: There are several commercially available instruments that rely on storage phosphor technology to image radioisotopes. Among these are the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the Molecular Imager (Bio-Rad, Hercules, CA).
2. Storage phosphor screens: Phosphor screens can be obtained directly from Molecular Dynamics or Bio-Rad, or from other companies, such as Fuji (Tokyo,

Japan) and Kodak (Rochester, NY). However, it has been our experience that the screens from different companies are of similar quality *(1)*. In our laboratory, we use both a Molecular Dynamics PhosphorImager SF interfaced to a networked Pentium PC and the Bio-Rad Molecular Imager interfaced to a networked PowerPC machine, but we have more experience with the Molecular Dynamics instrument for double-label analysis.

3. A 0.0014-in.-thick piece of copper with an area large enough to cover the phosphor screen being used is required to attenuate preferentially the ^{35}S signal for double-label experiments.
4. 96-Well plate with open bottoms.
5. Rapidly polymerizing polyacrylamide (same % T as the second-dimension gel). For example, add 80 µL of 10% APS and 40 µL of TEMED to 3 mL of 12% T solution. This recipe polymerizes in <30 s.
6. Scintillation counter.
7. Scintillation fluid.

3. Methods

Because the use of storage phosphor imaging technology for detection of ^{32}P- or ^{35}S-labeled proteins is straightforward *(2)*, this section will focus on the use of storage phosphor screen imaging in double-label image analysis of 2-D gels.

3.1. Sample Preparation

Proteins can be labeled by depleting intracellular phosphate and methionine levels. Subsequently, ^{32}P, ^{35}S, or both can be added to the cell culture and incorporated by cellular uptake. The length of time for the addition of label will depend on the type of cells being cultured. Prepare protein extracts as described in Chapters 3–11.

3.2. Preparation of Radiolabel Standards

For accurate quantitation, radioactive standards should be prepared (*see* **Note 1**).

1. Serially dilute an aliquot of radiolabeled protein extract over five orders of magnitude. Determine the precise radioactivity of the initial standard by scintillation counting.
2. For accurate quantitation, incorporate the standards into a polyacrylamide gel, and dry onto filter paper in a method similar to that used for the 2-D gel (*see* **Note 2**). Place a 96-well plate with open bottoms on a piece of parafilm.
3. Carefully pipet each of the desired standards (0–5 µL) into each well followed by 60 µL of a rapidly polymerizing polyacrylamide gel. Use the same percentage acrylamide as used for the second-dimension gel (*see* **Note 3**).
4. After polymerization, carefully remove the 96-well plate to yield a number of polyacrylamide plugs.
5. Vacuum-dry the polyacrylamide plugs on filter paper. This process yields a series of evenly spaced standards that can be exposed along with every 2-D gel.
6. Separately, add an aliquot of the stock ^{32}P-orthophosphate to colored ink at a dilution of 1:10,000 for use as a gel-orientation marker.

3.3. Storage Phosphor Screen Imaging

1. To detect ^{32}P-labeled proteins (without detecting ^{35}S label), place the dried 2-D gels alongside the radioactive standards for exposure (*see* Chapter 31). Apply 5 μL of the radioactive ink on at least three corners of the filter paper on which the gels are dried. These markers provide a common reference point to allow the precise overlapping of the 2-D images common to each gel.

2. On top of the marked 2-D gel filter, place a 0.0014-in. copper sheet followed by an erased imaging screen and expose (*see* **Note 4**). The copper sheet attenuates 99.9% of the ^{35}S signal, whereas the ^{32}P signal is attenuated by only 30% *(3)*. Exposure times can vary from several hours to a few days depending on the amount of label incorporated.

3. For double-label analysis, image the 2-D gels and standards with (^{32}P-labeled proteins) and without (total protein) the copper filter (*see* **Note 5**).

4. Analyze the images by printing out hard copy images onto translucent materials. Using the radioactive ink reference coordinates, precisely overlay the images (derived from the same 2-D gel). Alternatively, analyze the data using currently available software packages (*see* Chapter 40). A comparison of these two images will provide information on those spots corresponding to proteins that are phosphorylated.

4. Notes

1. Standards should be run in duplicate.

2. There is no need to fix the radiolabel standards chemically (proteins) into the polyacrylamide plugs. Fixation can lead to a decrease in the concentration of material present.

3. When using gradient gels in the second dimension, the selection of appropriate %T to match the variable quenching effect in the polyacrylamide gradient is not clear. We recommend either using several standards each at different %T concentrations or using an average concentration of polyacrylamide.

4. Storage phosphor plates should be stored protected from light, and erased before and after each use. The plates can be periodically cleaned using Kodak Intensifying Screen Cleaner.

5. A ratio of about 100:1 of ^{35}S:^{32}P cpm is optimal for maximizing differences between the filtered and nonfiltered images.

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33

Silver Staining of 2-D Electrophoresis Gels

Thierry Rabilloud

1. Introduction

Silver staining of polyacrylamide gels was introduced in 1979 by Switzer et al. *(1)* and rapidly gained popularity owing to its high sensitivity, ca. 100 times higher than staining with Coomassie blue. However, the first silver-staining protocols were not trouble-free. High backgrounds and silver mirrors were frequently experienced, with a subsequent decrease in sensitivity and reproducibility. This led many authors to suggest improved protocols, so that more than 100 different silver-staining protocols for proteins in polyacrylamide gels can be found in the literature. However, all of them are based on the same principle (*see* **refs.** *2* and *3* for details) and comprise more or less four major steps.

1. The first step is fixation, and aims at insolubilizing the proteins in the gels and removing the interfering compounds present in the 2-D gels (glycine, Tris, SDS, and carrier ampholytes present at the bottom of the gels).
2. The second step is sensitization, and aims at increasing the subsequent image formation. Numerous compounds have been proposed for this purpose. All these compounds bind to the proteins, and are also able either to bind silver ion, or to reduce silver ion into metallic silver, or to produce silver sulfide *(2,3)*. This sensitization step is sometimes coupled with the fixation step.
3. The third step is silver impregnation. Either plain silver nitrate or ammoniacal silver can be used.
4. The fourth and last step is image development. For gels soaked with silver nitrate, the developer contains formaldehyde, carbonate, and thiosulfate. The use of the latter compound, introduced by Blum et al. *(4)*, reduces dramatically the background and allows for thorough development of the image. For gels soaked with ammoniacal silver, the developer contains formaldehyde and citric acid. In this case, thiosulfate is better introduced at the gel polymerization step *(5)*. Background reduction by thiosulfate can also be achieved by brief incubation in thiosulfate prior to development *(6)* or by inclusion in the developer.

When the desired image level is obtained, development is stopped by dipping the gel in a stop solution, generally containing acetic acid and an amine to reach a pH of 7.0. Final stabilization of the image is achived by thorough rinsing in water to remove all the compounds present in the gel.

2. Materials

2.1. Equipment

1. Glass dishes or polyethylene food dishes: The latter are less expensive, have a cover, and can be easily piled up for multiple staining. They are, however, more difficult to clean, and it is quite important to avoid scratching of the surface, which will induce automatic silver deposition in subsequent stainings. Traces of silver are generally easily removed by wiping the plastic box with a tissue soaked with ethanol. If this treatment is not sufficient, stains are easily removed with Farmer's reducer (0.1% sodium carbonate, 0.3% potassium hexacyanoferrate [III], and 0.6% sodium thiosulfate). Thorough rinsing of the box with water and ethanol terminates the cleaning process.
2. Plastic sheets (e.g., the thin polycarbonate sheets sold by Bio-Rad [Hercules, CA] for multiple gel casting): Used for batch processing.
3. Reciprocal shaking platform: The use of orbital or three-dimensional movement shakers is not recommended.

2.2. Reagents

Generally speaking, chemicals are of standard pro-analysis grade.

1. Water: The quality of the water is of great importance. Water purified by ion-exchange cartridges with a resistivity >15 MΩ/cm is very adequate, whereas distilled water gives more erratic results.
2. Formaldehyde: Formaldehyde stands for commercial 37–40% formaldehyde. This is stable for months at room temperature. It should not be stored at 4°C, since this promotes polymerization and deposition of formaldehyde. The bottle should be discarded when a layer of polymer is visible at the bottom of the bottle.
3. Sodium thiosulfate solution: 10% (w/v) solution of crystalline sodium thiosulfate pentahydrate in water. Small volumes of this solution (e.g., 10 mL) are prepared fresh every week and stored at room temperature.
4. Glutaraldehyde: Glutaraldehyde is purchased as 50% solution and stored at 4°C for months. Microscopy-grade solutions are not required. However, good-quality glutaraldehyde is needed, and yellowish solutions should not be used. Good results are obtained with the Fluka brand (Buchs, Switzerland).
5. Ethanol: A technical grade of alcohol can be used, and 95% ethanol can be used instead of absolute ethanol, without any volume correction. The use of denatured alcohol is, however, **not** recommended.
6. Citric acid solution: 2 *M* citric acid, stored at room temperature for months.
7. Potassium acetate solution: 5 *M* potassium acetate. This is stable for months at room temperature.

8. Silver nitrate solution: 1 *N* silver nitrate. A 1 *N* silver nitrate solution (Fluka) is less expensive than solid-silver nitrate, and is stable for months if kept in a refrigerator (a black and cold place).
9. Sodium hydroxide solution: 1 *N* sodium hydroxide (Fluka).
10. Ammonium hydroxide solution: 5 *N* ammonium hydroxide (Aldrich, Milwaukee, WI). Ammonium hydroxide is kept in the refrigerator.

2.2.1. Solutions for Protocol 1

1. Fix solution I: 25% v/v ethanol, 4% v/v formaldehyde.
2. Sensitivity enhancing solution I: 0.05% (w/v) 2-7 Naphtalene disulfonate disodium salt (NDS) (Acros Chemicals, Geel, Belgium).
3. Ammoniacal silver solution: To prepare 500 mL of this solution (sufficient for a batch of 4 gels), place 480 mL of water in a flask under strong magnetic agitation. Add successively 7.5 mL of 1 *N* sodium hydroxide, 7.5 mL of 5 *N* ammonium hydroxide, and 12 mL of 1 *N* silver nitrate. On addition of silver nitrate, a transient brown precipitate forms, which should redissolve within a few seconds (*see* **Note 3**). Only clear solutions should be used.
4. Development solution I: 1 mL of 37% formaldehyde and 180 µL of 2 *M* citric acid/L (*see* **Note 4**).
5. Stop solution I: 20 mL acetic acid and 5 mL ethanolamine/L.

2.2.2. Solutions for Protocol 2

1. Fix solution II: 5% acetic acid, 30% ethanol.
2. Sensitivity-enhancing solution II: 2 mL of 10% thiosulfate/L.
3. Silver stain solution II: 0.7 mL of 37% formaldehyde and 12.5 mL of 1 *N* silver nitrate/L.
4. Development solution II: 30 g anhydrous potassium carbonate, 250 µL 37% formaldehyde, and 125 µL 10% thiosulfate/L (*see* **Note 9**).
5. Stop solution II: 40 g of Tris and 20 mL of acetic acid/L.

2.2.3. Solutions for Protocol 3

1. Fix solution III: 5% acetic acid/30% ethanol.
2. Sensitivity-enhancing solution III: 0.5 *M* potassium acetate, 0.5% glutaraldehyde, 25% ethanol, and 3 g potassium tetrathionate/L (*see* **Notes 10** and **11**).
3. Silver stain solution III: 0.5 mL 37% formaldehyde and 12.5 mL 1 *N* silver nitrate/L.
4. Development solution III: 30 g anhydrous potassium carbonate, 250 µL 37% formaldehyde and 125 µL 10% thiosulfate/L (*see* **Note 9**).
5. Stop solution III: 40 g of Tris and 20 mL of acetic acid/L.

2.2.4. Solutions for Protocol 4

1. Fix solution IV: 5% acetic acid/30% ethanol.
2. Sensitivity-enhancement solution IV: 0.5 *M* potassium acetate, 0.5% glutaraldehyde, 25% ethanol, and 3 g potassium tetrathionate/L (*see* **Notes 10** and **11**).

3. Silver stain solution IV: 0.6 mL 37% formaldehyde, 12.5 mL 1 *N* silver nitrate, and 2.5 g HEPES hemisodium salt (Sigma, St. Louis, MO)/L (*see* **Note 12**).

4. Development solution IV: 30 g anhydrous potassium carbonate, 300 μL 37% formaldehyde, and 125 μL 10% thiosulfate/L (*see* **Note 9**).

5. Stop solution IV: 40 g of Tris and 20 mL of acetic acid/L.

3. Methods

3.1. General Practice

Batches of gels (up to 4 gels/box) can be stained. For a batch of three or four medium-sized gels (e.g., 160 × 200 × 1.5 mm), 1 L of the required solution is used, which corresponds to a solution/gel volume ratio of at least 5. Five hundred milliliters of solution are used for one or two gels. Batch processing can be used for every step longer than 5 min, except for image development, where 1 gel/box is required. For steps shorter than 5 min, the gels should be dipped individually in the corresponding reagent(s).

For changing solutions, the best way is to use a plastic sheet. This is pressed on the pile of gels with the aid of a gloved hand. Inclining the entire setup allows emptying the box while keeping the gels in it. The next solution is poured with the plastic sheet in place, which prevents the flow from folding or breaking the gels. The plastic sheet is removed after the solution change and kept in a separate box filled with water until the next solution change. This water is changed after each complete round of silver staining.

When gels must be handled individually, the are manipulated with gloved hands. The use of powder-free, nitrile gloves is strongly recommended, since standard gloves are often the cause of pressure marks.

Except for development or short steps, where occasional hand agitation of the staining vessel is convenient, constant agitation is required for all the steps. A reciprocal ("ping-pong") shaker is used at 30–40 strokes/min.

Four different silver-staining protocols are detailed below. The rationale for choosing one of them according to the constraints brought by the precise 2-D protocol used and the requisites of the experimenter are described in **Note 1**.

3.2. PROTOCOL 1: Silver Staining with Ammoniacal Silver

This protocol is based on the original protocol of Eschenbruch and Bürk *(7)*, with modifications *(5,8,9)*. For optimal results, alterations must be brought at the level of gel casting. Piperazine diacrylamide is used as crosslinker in place of *bis* (in a weight-to-weight substitution) *(8)*, and thiosulfate is added at the gel-polymerization step *(5)*. Practically, the initiating system is composed of 1 μL of TEMED, 10 μL of 10% sodium thiosulfate solution, and 10 μL of 10% ammonium persulfate solution/mL of gel mix. This ensures correct gel formation and gives minimal background on staining.

After electrophoresis, silver staining processes are as follows:

1. Unmold the gels in water, and let rinse for 5–10 min.
2. Soak gels in fix solution I for 1 h (*see* **Note 2**).
3. Rinse 2 × 15 min in water.
4. Sensitize overnight in sensitivity-enhancing solution I.
5. Rinse 6 × 20 min in water.
6. Impregnate for 30-60 min in the ammoniacal silver solution.
7. Rinse 3 × 5 min in water.
8. Develop image (5–10 min) in development solution I.
9. Stop development in stop solution I. Leave in this solution for 30–60 min.
10. Rinse with water (several changes) prior to drying or densitometry.

3.3. PROTOCOL 2: Fast Silver Staining

This protocol is based on the protocol of Blum et al. *(4)*, with modifications *(9)*.

1. Soak the gels in fix solution II for at least 3 × 30 min (*see* **Note 5**).
2. Rinse in water for 3 × 10 min.
3. To sensitize, soak gels for 1 min (one gel at a time) in sensitivity-enhancing solution II.
4. Rinse 2 × 1 min in water (*see* **Note 6**).
5. Impregnate for at least 30 min in silver solution II (*see* **Note 7).**
6. Rinse in water for 5–15 s (*see* **Note 8**).
7. Develop image (10–20 min) in development solution II (*see* **Note 9**).
8. Stop development (30–60 min) in stop solution II.
9. Rinse with water (several changes) prior to drying or densitometry.

3.4. PROTOCOL 3: Silver Staining for Supported Gels (3)

For this protocol, only 1 gel can be stained/vessel.

1. Fix the gels in fix solution III (3 × 30 min).
2. Sensitize for 30–60 min in sensitivity-enhancement solution III.
3. Rinse in water (4 × 15 min).
4. Impregnate for 30–60 min with silver stain solution III.
5. Rinse with water for 5–10 s (*see* **Note 8**).
6. Develop image (10–20 min) in development solution III (*see* **Note 9**).
7. Stop development (30–60 min) in stop solution III.
8. Rinse with water (several changes) prior to drying or densitometry.

3.5. PROTOCOL 4: Long Silver Nitrate Staining for Free-Floating Gels (3)

1. Fix the gels in fix solution IV (3 × 30 min).
2. Sensitize overnight in sensitivity enhancement solution IV.
3. Rinse in water (6 × 20 min).
4. Impregnate for 1–2 h with silver in a silver solution IV.

5. Rinse with water for 5–10 s (*see* **Note 8**).
6. Develop image (10–20 min) in development solution IV (*see* **Note 9**).
7. Stop development (30–60 min) in stop solution IV.
8. Rinse with water (several changes) prior to drying or densitometry.

4. Notes

1. From the rather simple theoretical bases described in **Subheading 1.**, more than 100 different protocols were derived. The changes from one protocol to another are present either in the duration of the different steps or in the composition of the solutions. The main variations concern either the concentration of the silver reagent, or the nature and concentration of the sensitizers. Only a few comparisons of silver-staining protocols have been published *(9,10)*. From these comparisons, selected protocols have been proposed in the former sections. The choice of a protocol will depend on the constraints of the experimental setup and of the requisites of the experimenter (speed, reproducibility, and so forth). The following guidelines can be suggested.

   a. The maximum sensitivity is not widely different from one protocol to another. The main differences are in the uniformity of the staining from one protein to another, the reproducibility of the staining, the speed of the method, and its adaptation to the various 2-D protocols.

   b. Generally speaking, methods using ammoniacal silver give very uniform results, with minimal color effects. They are by far more sensitive than silver nitrate-based methods for the staining of basic proteins, and are therefore strongly recommended for 2-D gels with very wide pH gradients. However, these methods suffer from a number of minor drawbacks, which prevent their universal use.

   c. The silver reagent is very sensitive to the ammonia concentration. Since ammonia is highly volatile, this introduces problems for the long-term reproducibility of the method. This problem can be alleviated to a large extent by the use of commercial titrated ammonia solutions.

   d. Ammoniacal silver is not compatible with all SDS gel systems. Systems using Tricine or Bicine as trailing ions are **not** compatible with ammoniacal silver staining.

   e. Ammoniacal silver staining is not recommended for gels supported by a plastic film. Silver mirrors are frequently encountered in this case.

   f. Optimal protocols for ammoniacal silver staining (e.g., Protocol 1 in **Subheading 3.2.**) are generally time-consuming. In addition, optimal results are obtained with the use of homemade gels, containing PDA as a crosslinker *(8)* and with thiosulfate included at the gel polymerization step *(5)*. This prevents the use of commercial gels. Moreover, these protocols give best results when aldehydes (formaldehyde or glutaraldehyde) are used as fixers/sensitizers. This prevents any recovery of the silver-stained protein for subsequent use (e.g., mass spectrometry fingerprinting). This drawback can be, however, alleviated (*see* **Note 2**), at the expense of the uniformity of the staining (hollow spots can be experienced).

In conclusion, ammoniacal silver staining, as described in protocol 1 and in its variations in **Note 2**, is the method of choice if staining uniformity is the key parameter (especially with basic proteins), and if the experimenter is prepared to face the constraints mentioned above. In the other cases, staining with silver nitrate will be the method of choice. A good compromise among speed, sensitivity, and widespread applicability is offered by the staining method described in protocol 2. This method is applicable to all SDS (and also IEF) gel systems described to date, either on free-floating or supported gels. Subsequent digestion of the protein in the gel and peptide extraction are feasible. The main drawbacks of the method are the weak sensitivity for basic proteins and some variability from one experiment to another. The latter problem arises from the short steps (1 min or less) of the protocol, which are difficult to keep very reproducible from one experiment to another. In addition, these short steps make the protocol rather cumbersome when large series of gels are to be stained. If these latter drawbacks are considered too prominent, longer, more reproducible, silver nitrate stains are described in protocol 3 for supported gels and in protocol 4 for free-floating gels. These two protocols offer maximal reproducibility. Their only limitations lie in some color effects (lighter colors than with ammoniacal silver) and in weak staining of basic proteins.

2. Other fixation processes can be used. For gels running overnight, a shorter process can be used. Fix the gel in 5% acetic acid and 30% ethanol for 3 × 30 min, and then in water for 10 min. Fix for 30–60 min in a solution containing 1% glutaraldehyde and 0.5 *M* potassium acetate. Rinse 2 × 15 min in water, 2 × 30 min in 0.05% NDS, and 4 × 15 min in water. Return to standard protocol at **step 6** in **Subheading 3.2.** (silver impregnation). When aldehydes are to be avoided, fix directly in a solution containing 5% acetic acid, 30% ethanol, and 0.05% NDS *(11)*. If a short fixation (<3 h) is performed, rinse 6 × 10 min in water. If a long fixation (overnight) is performed, rinse 6 × 20 min in water. Then proceed to **step 6** in **Subheading 3.2.**

3. The composition of the ammoniacal silver solution has an important influence on the final sensitivity. The ammonia/silver mol ratio is in fact the key parameter *(7)*. The solution given in this protocol has an ammonium/silver molar ratio of 3.1, which ensures maximal sensitivity, but less stability of the solution. If a brown precipitate remains in the solution, this means that the ammonia solution is no longer concentrated enough. The best remedy is to discard the ammoniacal silver solution and to prepare a new one with a new bottle of 5 *N* ammonium hydroxide. If this is not possible, add small aliquots of ammonia to the precipitated ammoniacal silver solution until the solution becomes clear. The sensitivity will be, however, lesser than usual. If reduced sensitivities are required, increase the ammonium hydroxide concentration by a factor of 1.3–2. This will progressively decrease the sensitivity.

4. In a standard analytical 2-D gel loaded with 50–100 μg of protein, the first major spots should begin to appear within 1 min. Delayed appearance indicates lower than expected sensitivity, but is observed when aldehydes have not been used in

the fixing process. In the latter case, sensitivity is restored by a longer development. The developer should be altered if no thiosulfate is present in the gel (e.g., use of ready-made gels). To prevent the rapid appearance of background, add 10 μL of 10% thiosulfate/L of developer. The maximum sensitivity will not be altered by this variation. However, some spots will show a lighter color or give hollow spots.

5. The fixation process can be altered if needed. The figures indicated in the protocol are the minimum times. Gels can be fixed without any problem for longer periods. For example, gels can be fixed overnight, with only one solution change. For ultra-rapid fixation, the following process can be used *(12)*:
   a. Fix in 10% acetic acid/40% ethanol for 10 min, and then rinse for 10 min in water.
   b. Postfix in 0.05% glutaraldehyde/40% ethanol and 100 μL/L 37% formaldehyde for 5 min.
   c. Rinse in 40% ethanol for 2 × 10 min and then in water for 2 × 10 min. Proceed to **step 3** in **Subheading 3.3.**

6. The optimal setup for sensitization is the following. Prepare four staining boxes containing respectively, the sensitizing thiosulfate solution, water (two boxes), and the silver nitrate solution. Put the vessel containing the rinsed gels on one side of this series of boxes. Take one gel out of the vessel, and dip it in the sensitizing and rinsing solutions (1 min in each solution). Then transfer to silver nitrate. Repeat this process for all the gels of the batch. A new gel can be sensitized while the former one is in the first rinse solution, provided that the 1-min time is kept (use a bench chronometer). When several batches of gels are stained on the same day, it is necessary to prepare several batches of silver solution. However, the sensitizing and rinsing solutions can be kept for at least three batches, and probably more.

7. Gels can be impregnated with silver for at least 30 min and at most 2 h without any change in sensitivity or background.

8. This very short step is intended to remove the liquid film of silver solution brought with the gel.

9. When the gel is dipped in the developer, a brown microprecipitate of silver carbonate should form. This precipitate must be redissolved to prevent deposition and background formation. This is simply achieved by immediate agitation of the box. Do not expect the appearance of the major spots before 3 min of development. The spot intensity reaches a plateau after 15–20 min of development, and then background appears. Stop development at the beginning of background development. This ensures maximal and reproducible sensitivity.

10. The sensitization solution is prepared as follows. Dissolve potassium tetrathionate in water (half the desired final volume). After complete dissolution, add the required volumes of concentrated potassium acetate, ethanol, and glutaraldehyde. Fill up to the final volume with water.

11. If the use of aldehydes is to be avoided, the gels can be sensitized in a tetrathionate solution without aldehydes. For short sensitization (<1 h, just omit the glutaraldehyde). For overnight sensitization, use a solution containing 3 g/L potassium

tetrathionate, 5% acetic acid, and 25% ethanol. Rinses are the same as in the standard protocol. Compared to the standard protocol, lighter colors are obtained with the aldehyde-free protocol.

12. The silver solution is prepared as follows (for 1 L). Put 990 mL water in a clean glass vessel with magnetic stirring. Add 2.5 g HEPES hemisodium salt and 600 μL of formaldehyde, and let dissolve. Just prior to use, add 12.5 mL 1 *N* silver nitrate. If the glassware is not perfectly clean (e.g., detergent remnants), the solutions discolors, and a silver mirror may form. Should this happen, discard the solution, and prepare a fresh one. If possible, the use of disposable plasticware is recommended.

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34

Staining of Preparative 2-D Gels

Coomassie Blue and Imidazole-Zinc Negative Staining

Neil M. Matsui, Diana M. Smith-Beckerman, and Lois B. Epstein

1. Introduction

The identification of proteins using preparative gel electrophoresis and mass spectrometry requires reversible staining of relatively thick (1–1.5 mm) polyacrylamide gels. We have found that staining with colloidal Coomassie brilliant blue G-250 or negative staining with imidazole-zinc yields high-resolution stains (**Fig. 1**) that are compatible with subsequent mass spectrometric analysis (*see* **Notes 1** and **5**).

Coomassie blue G-250 complexes with basic amino acids, such as arginine, tyrosine, lysine, and histidine *(1)*. Neuhoff's stain *(2)* is sensitive in the detection of protein, and at the same time produces very low background staining (*see* **Note 2**). The low background staining is attributed to the colloidal properties of the stain that prevents the Coomassie dye from penetrating the gel.

The imidazole-zinc stain was developed by Ortiz et al. *(3–5)*. Since it is a negative stain, the background and not the protein is stained. Regions of the gel that are not occupied by protein allow the precipitation of a SDS-imidazole-zinc complex that forms the opaque white background. Regions of the gel that contain protein appear clear. This stain is almost as sensitive as most silver stains and requires only 30 min to complete (*see* **Note 2**). In addition, fixation of proteins to the gel is not needed, there is no interaction of the stain with the protein, and complete destaining of the matrix can be achieved.

Here we describe both methods, modified for use with preparative 2-D gels and mass spectrometry (*see* **Notes 1** and **5**). They are also suitable for analytical 2-D gels or 1-D SDS-PAGE gels. For thinner (<1 mm) gels, times of incubation may be decreased for the imidazole-zinc negative stain.

2. Materials

All solutions should be prepared in clean glassware with deionized, distilled water.

2.1. Coomassie Blue G-250 Staining Reagents

1. Gel fixative solution: 45% (v/v) methanol, 1% (v/v) acetic acid.
2. Coomassie blue G-250 stain: 17% (w/v) $(NH_4)_2SO_4$, 34% (v/v) methanol, 0.5% (v/v) acetic acid, 0.1% (w/v) Coomassie blue G-250 (Serva, New York, NY).

2.2. Imidazole-Zinc Negative Staining Reagents

1. Sodium carbonate solution: 1% sodium carbonate.
2. Imidazole-SDS solution: 200 mM imidazole, 0.1% (w/v) SDS.
3. Zinc acetate solution: 0.1 M zinc acetate. Filter any particles using a Whatman no. 1 filter.

3. Method

All solutions and gels should be handled with gloves, and all steps carried out at room temperature in staining dishes on a rocking or rotating platform.

3.1. Coomassie Blue G-250 Staining (2,6)

Fixation of the gel is done immediately after electrophoresis (*see* **Note 3**). Note, however, that gel fixation is not a required step for this stain.

1. Place the gel in the fixative solution for 20 min or more (*see* **Note 4**). Gels can be fixed overnight.
2. Decant the fixative solution.
3. Add the Coomassie blue G-250 stain. Incubate overnight (18–24 h).
4. Decant the stain.
5. Wash several times in water, approx 20 min each, until the desired contrast is achieved. Proteins appear as blue regions on a clear background. Warm water (45–55°C) facilitates washing.
6. The gel can be subjected to densitometric scanning, then wrapped in plastic wrap, and stored in a sealed tub or Ziploc bag at 4°C.

3.2. Imidazole-Zinc Negative Staining (3,7)

For the imidazole-zinc negative staining, no fixation is necessary. Gels are typically stained immediately after electrophoresis (*see* **Note 3**).

Fig. 1. *(opposite page)* Preparative 2-D gels stained by the imidazole-zinc negative stain (left) and colloidal Coomassie blue G-250 stain (right). Gels were scanned by a Computing Densitometer (Molecular Dynamics, CA). Top: 1 mg of protein from ME-180 cervical carcinoma cells was separated by carrier ampholyte IEF and 11% SDS-PAGE, and then stained using the imidazole-zinc stain. Bottom: 1 mg of protein from A375 human melanoma cells was separated by carrier ampholyte IEF and 11% SDS-PAGE, and then stained using the colloidal Coomassie blue G-250.
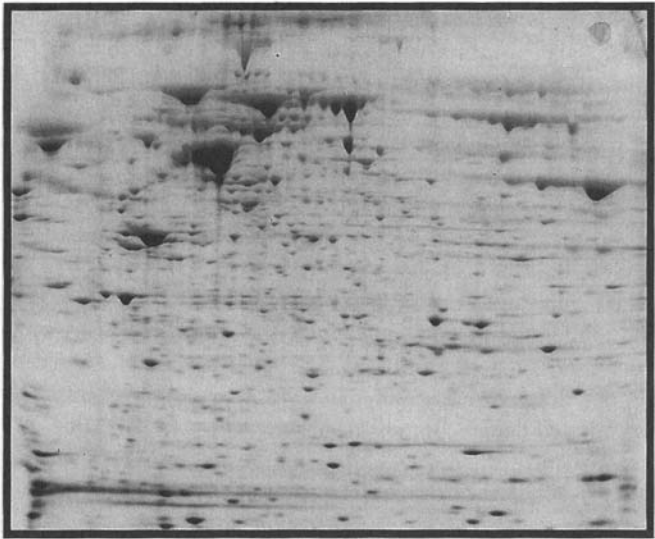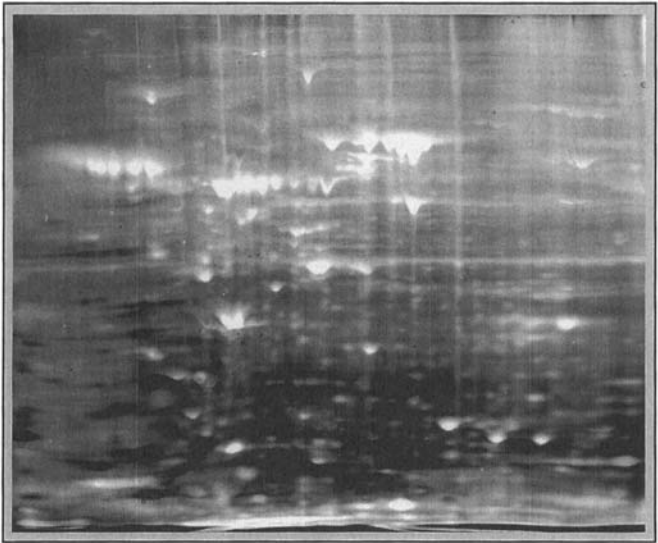
1. Place the gel in the sodium carbonate solution for 5 min (*see* **Note 4**). Gels can be left in this solution for slightly longer periods of time.
2. Decant the sodium carbonate solution.
3. Add imidazole-SDS solution. Incubate for 15–30 min.
4. Decant the imidazole-SDS solution.
5. Wash the gel twice in water for 30–45 s each wash.
6. Add zinc acetate solution. Vigorously swirl to get even staining.
7. Staining over a dark background is ideal for observing stain development. When the desired contrast is achieved, decant the zinc acetate solution. Proteins appear as clear regions in an opaque white background. Staining typically takes 0.5–3 min.
8. Wash the gel three times in water for 1–2 min each wash.
9. The gel can be subjected to densitometric scanning, then wrapped in plastic wrap, and stored in a sealed tub or Ziploc bag at 4°C.

4. Notes

1. The Fast Stain (Zoion, Newton, MA) method is a highly purified form of Coomassie blue R-250. This stain has been used in our laboratory with good results and is compatible with mass spectrometry.
2. There are differences in sensitivity and times of staining for the three stains described here. Our experience is that the imidazole-zinc stain is nearly as sensitive as silver staining (1–10 ng of BSA), and requires about 30 min to complete. The colloidal Coomassie blue G-250 stain is three- to fivefold less sensitive than the imidazole-zinc and requires 1 d to complete. The fast stain is only slightly less sensitive than the Coomassie blue G-250 stain and requires 1 h to complete. Although the imidazole-zinc negative stain is the most sensitive and the most rapid of these stains, they require a dark background to view. Also, imidazole-zinc-stained gels appear to be slightly more brittle and prone to dryness over long-term storage. In our hands, the standard Coomassie blue R-250 stain is less sensitive than any of these stains, and also has the problems of a high level of background staining and large amounts of alcohol waste.
3. Rough handling of large 2-D gels can cause fractures and breaks. If such problems occur, replace acrylamide with Duracryl (ESA, Chelmsford, MA) in SDS-PAGE separating gels.
4. A staining dish should be chosen, such that it is slightly larger than the gels that are being stained. Each gel, especially large format gels, should be stained in separate staining dishes. Large plastic staining dishes are used in our laboratory, but glass dishes are also appropriate.
5. We have obtained good-quality peptide mass spectra and peptide sequence spectra from proteins of one to four replicate spots from gels stained with each of the methods described in this chapter. These spots were excised from gels loaded with 1 mg of cell lysates from A375 melanoma (data not shown) and ME-180 cervical carcinoma cells *(8)*. Coomassie blue can be removed with organic solutions used during in-gel trypsin digestion *(9)*. For imidazole-zinc-stained gel plugs, it is suggested, but not necessary, to destain gel plugs with 2% citric acid prior to in-gel trypsin digestion. Note that modifications can be made to the silver

stain protocol as well to permit subsequent mass spectrometry *(10)*. Silver-staining protocols for mass spectrometry, however, are still under development.

Acknowledgments

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35

Electroblotting of Proteins from 2-D Polyacrylamide Gels

Michael J. Dunn

1. Introduction

The ability of two-dimensional electrophoresis (2-DE) to separate complex mixtures of proteins, such as represented by cells, tissues, and even whole organisms, has been recognized for more than 20 yr *(1)*. Using "standard"-format (around 20 × 20 cm) 2-D gels, the method is capable of routinely separating 2000 proteins from whole-cell and tissue extracts. The resolution capacity can be extended significantly (up to 5000–10,000 proteins) using large-format (40 × 30 cm) 2-D gels *(2)*. Over the last 20 yr, 2-DE has been used primarily as an analytical tool for the characterization of proteins by their charge (pI), size (M_r), and relative abundance. Specialized computer software has been developed for the qualitative and quantitative analysis of 2-DE protein patterns *(3)*, and these systems have been used to construct several comprehensive databases of protein expression in a variety of cell types and tissues (for examples, *see* **ref. 4**).

A major obstacle to the full exploitation of such databases has been that the method provides no direct clues to the identity or function of the separated components. Until relatively recently, only time-consuming and laborious methods, such as specific staining methods, cellular subfractionation, and coelectrophoresis with purified proteins, were available for protein identification following 2-DE. A major advance was made with the development of protein blotting techniques, based on those developed by Southern *(5)* for the analysis of electrophoretic separations of DNA. In these procedures, following separation by 2-DE, the proteins are transferred ("blotted") onto the surface of an inert membrane, such as nitrocellulose. When immobilized in this way, the proteins are readily accessible
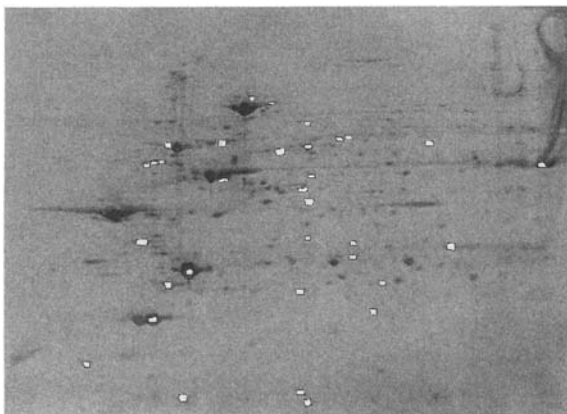
Fig. 1. PVDF (FluoroTrans) Western electroblot transfer of 300 μg of dog heart proteins separated by 2-DE and stained with Coomassie brilliant blue R-250. The white areas indicate protein spots that have been excised for chemical characterization.

to interaction with probes, such as polyclonal antibodies and monoclonal antibodies (MAbs) or other ligands specific for the protein being analyzed.

More recently, 2-DE has become the method of choice for the micropreparative purification of proteins for subsequent chemical characterization and several projects aimed at the characterization of all the proteins expressed by the genome of an organism, the so-called proteome, have been initiated *(6)*. In this approach, proteins separated by 2-DE are blotted onto an appropriate membrane support, the protein profile visualized using a suitable total protein stain, and the protein spot of interest is excised (**Fig. 1**). The protein, while still on the surface of the inert membrane support, can then be subjected to a battery of sensitive microchemical characterization techniques, including N-terminal and internal amino acid sequencing, amino acid compositional analysis, peptide profiling, and mass spectrometry *(7)*.

The most efficient method for the transfer of proteins separated by 2-DE to membranes is the application of an electric field perpendicular to the plane of the gel. This technique of electrophoretic transfer, first described by Towbin et al. *(8)*, is also known as Western blotting. Two types of apparatus are in routine use for electroblotting. In the first approach (known as "tank" blotting), the sandwich assembly of gel and blotting membrane is placed vertically between two platinum wire electrode arrays contained in a tank filled with blotting buffer *(9)*. The disadvantages of this technique are that:

1. A large volume of blotting buffer must be used.
2. Efficient cooling must be provided if high current settings are employed to facilitate rapid transfer.

3. The field strength applied (V/cm) is limited by the relatively large interelectrode distance.

In the second type of procedure (known as "semidry" blotting), the gel-blotting membrane assembly is sandwiched between two horizontal plate electrodes, typically made of graphite *(10)*. The advantages of this method are that:

1. Relatively small volumes of transfer buffer are used.
2. Special cooling is not usually required, although the apparatus can be run in a cold room if necessary.
3. A relatively high field strength (V/cm) is applied owing to the short interelectrode distance resulting in faster transfer times.

In the following sections, both tank and semidry electroblotting methods for recovering proteins separated by 2-DE will be described.

2. Materials

2.1. Electroblotting

Prepare all buffers from analytical-grade reagents and dissolve in deionized water. The solutions should be stored at 4°C and are stable for up to 3 mo.

1. Blotting buffers are selected empirically to give the best transfer of the protein(s) under investigation. The following compositions are commonly used:
   a. For immunoprobing of proteins with p*I*s between pH 4.0 and 7.0: 20 m*M* Tris, 150 m*M* glycine, pH 8.3. To prepare the solution, dissolve 2.42 g Tris base and 11.26 g glycine, and make up to 1 L (*see* **Note 1**). The pH of the buffer is pH 8.3 and should not require adjustment.
   b. For chemical characterization of proteins with p*I*s between pH 4.0 and 7.0 (*see* **Note 2**): 50 m*M* Tris, 50 m*M* borate, pH 8.5 *(11)*. To prepare the solution, dissolve 6.06 g Tris base and 3.09 g boric acid in water, adjust the solution to pH 8.5 with 10 *N* sodium hydroxide, and make up to 1 L (*see* **Note 1**).
   c. For proteins with p*I*s between pH 6.0 and 10.0: 10 m*M* 3-[cyclohexyl-amino]-1-propanesulfonic acid (CAPS), pH 11.0 *(12)*. To prepare the solution, dissolve 2.21 g CAPS in water, adjust the solution to pH 11.0 with 10 *N* sodium hydroxide, and make up to 1 L (*see* **Note 1**).
2. Filter paper: Whatman 3MM filter paper cut to the size of the gel to be blotted.
3. Transfer membrane:
   a. For immunoprobing: Hybond-C Super (Amersham Pharmacia Biotech, Aylesbury, UK) cut to the size of the gel to be blotted (*see* **Note 3**).
   b. For chemical characterization: FluoroTrans (Pall, Harant, UK) cut to the size of the gel to be blotted (*see* **Note 4**).
4. Electroblotting equipment: A number of commercial companies produce electroblotting apparatus and associated power supplies. For tank electroblotting, we use the TE 42 Transphor Unit from Hoefer Scientific Instruments, whereas for semidry electroblotting, we use the NovaBlot apparatus from Amersham Pharmacia Biotech.

5. Rocking platform.
6. Plastic boxes for gel incubations.

3. Method

3.1. Semidry Blotting

1. Following separation of the proteins by gel electrophoresis, place the gel in equilibration buffer, and gently agitate for 30 min at room temperature (*see* **Note 5**).
2. Wet the lower (anode) plate of the electroblotting apparatus with deionized water.
3. Stack six sheets of filter paper wetted with blotting buffer on the anode plate and roll with a glass tube to remove any air bubbles.
4. Place the prewetted transfer membrane (*see* **Note 6**) on top of the filter papers, and remove any air bubbles with the glass tube.
5. Place the equilibrated gel on top of the blotting membrane and ensure that no air bubbles are trapped.
6. Apply a further six sheets of wetted filter paper on top of the gel, and roll with the glass tube.
7. Wet the upper (cathode) plate with deionized water, and place on top of the blotting sandwich.
8. Connect the blotter to power supply, and transfer at 0.8 mA/cm^2 of gel area (*see* **Note 7**) for 1 h at room temperature (*see* **Note 8**).

3.2. Tank Blotting

1. Following separation of the proteins by gel electrophoresis, place the gel in equilibration buffer, and gently agitate for 30 min at room temperature (*see* **Note 6**).
2. Place the anode side of the blotting cassette in a dish of blotting buffer.
3. Submerge a sponge pad taking care to displace any trapped air and place on top of the anodic side of the blotting cassette.
4. Place two pieces of filter paper onto the sponge pad, and roll with a glass tube to ensure air bubbles are removed.
5. Place the prewetted transfer membrane (*see* **Note 7**) on top of the filter papers, and remove any air bubbles with the glass tube.
6. Place the equilibrated gel on top of the blotting membrane, and ensure that no air bubbles are trapped.
7. Place a sponge pad into the blotting buffer taking care to remove any trapped air bubbles, and then place on top of the gel.
8. Place the cathodic side of the blotting cassette on top of the sponge, and clip to the anode side of the cassette.
9. Remove the assembled cassette from the dish, and place into the blotting tank filled with transfer buffer.
10. Connect to the power supply and transfer for 6 h (1.5-mm-thick gels) at 500 mA at 10°C (*see* **Note 8**).

4. Notes

1. Methanol (10–20%, v/v) is often added to transfer buffers as it removes SDS from protein–SDS complexes and increases the affinity of binding of proteins to

nitrocellulose. However, methanol acts as a fixative and reduces the efficiency of protein elution, so that extended transfer times must be used. This effect is worse for high-mol-wt proteins, so that methanol is best avoided if proteins >100 kDa are to be transferred.

2. The use of transfer buffers containing glycine or other amino acids must be avoided for proteins to be subjected to microchemical characterization.

3. Nitrocellulose is the most popular support for electroblotting, since it is compatible with most general protein stains, is relatively inexpensive, and has a high protein-binding capacity (249 µg/cm2 *[13]*). Hybond-C Super is a 0.45-µm pore size supported nitrocellulose membrane, which is more robust than an unsupported matrix. Nitrocellulose membranes of smaller pore size are available (0.1 and 0.2 µm) and can give better retention of small proteins (<1500 kDa). Polyvinylidene difluoride (PVDF) membranes, which have a high mechanical strength and a binding capacity similar to that of nitrocellulose (172 µg/cm^2 *[13]*), are compatible with most immunoblotting protocols.

4. Nitrocellulose membranes are not compatible with the reagents and organic solvents used in automated protein sequencing. A range of PVDF-based (FluoroTrans, Pall; ProBlott, Applied Biosystems; Immobilon-P and Immobilon-CD, Millipore; Westran, Schleicher and Schuell; Trans-Blot, Bio-Rad), glass fiber-based (Galssybond, Biometra; PCGM-1, Janssen Life Sciences), and polypropylene-based (Selex 20, Schleicher and Schuell) membranes have been developed to overcome this problem *(12,14–16)*. We have found that PVDF-based membranes have a higher protein-binding capacity, and result in better average repetitive and initial sequencing yields *(11)*. Nitrocellulose can be used as a support in applications, such as internal amino acid sequence analysis and peptide profiling, where the protein band or spot is subjected to proteolytic digestion prior to characterization of the released peptides.

5. Gels are equilibrated in blotting buffer to remove excess SDS and other reagents that might interfere with subsequent analysis (e.g., glycine). This step also minimizes swelling effects during protein transfer. Equilibration may result in diffusion of zones and reduced transfer efficiencies of high-mol-wt proteins. It is important to optimize the equilibration time for the protein(s) of interest.

6. Nitrocellulose membranes can be wetted with blotting buffer, but PVDF-based membranes must first be wetted with methanol prior to wetting with the buffer.

7. The maximum mA/cm^2 of gel area quoted applies to the apparatus we have used. This should be established from the manual for the particular equipment available.

8. Blotting times need to be optimized for the particular proteins of interest and according to gel thickness. Larger proteins usually need a longer transfer time, whereas smaller proteins require less time. Proteins will also take longer to be transferred efficiently from thicker gels. The transfer time cannot be extended indefinitely (>3 h) using the semidry technique, since the small amount of buffer used will evaporate. If tank blotting is used, the transfer time can be extended almost indefinitely (>24 h) providing that the temperature is controlled.

Acknowledgment

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36

Detection of Total Proteins
on Western Blots of 2-D Polyacrylamide Gels

Michael J. Dunn

1. Introduction

The ability of two-dimensional electrophoresis (2-DE), based on the original method developed more than 20 years ago *(1)*, to separate simultaneously up to several thousand proteins using large-format gels *(2)* has made it the method of choice for the analysis of protein expression in complex biological systems, such as whole cells, tissues, and organisms. Initially, 2-DE only yielded information on the charge (pI), size (M_r), and relative abundance of the separated proteins. However, in recent years, a variety of methods have been developed that make it possible to identify and characterize proteins separated by 2-DE. Many of these methods depend on the technique of Western electroblotting in which proteins separated by 2-DE are transferred ("blotted") by the application of an electric field perpendicular to the plane of the gel onto the surface of an inert membrane, such as nitrocellulose *(3)*. Methods for electroblotting of protein from 2-DE gels are described in Chapter 35.

Proteins immobilized in this way are readily accessible to interaction with probes, such as polyclonal antibodies and monoclonal antibodies (MAbs) or other ligands specific for the protein being analyzed. This approach has been used extensively for the identification of proteins separated by 2-DE. More recently, 2-DE has become the method of choice for the micropreparative purification of proteins for subsequent chemical characterization and several projects aimed at the characterization of all the proteins expressed by the genome of an organism, the so-called proteome, have been initiated *(4)*. In this approach the protein, while still on the surface of the inert membrane support, can be analyzed by several sensitive chemical characterization techniques,

Table 1
Methods for the Detection of Total Proteins
on Western blot Transfers of 2-DE Gels in Order of Increasing Sensitivity[a]

Detection reagent	Approximate sensitivity, protein per spot	Matrix compatibility
Ponceau S	5 μg	NC, PVDF
Fast green FC	5 μg	NC, PVDF
Amido black 10B	1 μg	NC, PVDF
Coomassie brilliant blue R-250	500 ng	PVDF
Pyrogallol-red molybdate	500 ng	NV, PVDF
Immobilon-CD stain	500 ng	Immobilon-CD
Instaview Nitrocellulose	250 ng	NC, PVDF
India Ink	100 ng	NC, PVDF
Double metal chelate stain	50 ng	NC, PVDF
Colloidal iron	30 ng	NC, N, PVDF
In situ biotinylation + HRP + avidin	30 ng	NC, N, PVDF
Colloidal gold	4 ng	NC, PVDF

[a]NC, nitrocellulose; PVDF, polyvinylidene difluoride; N, nylon; HRP, horseradish peroxidase.

including N-terminal and internal amino acid sequencing, amino acid compositional analysis, peptide profiling, and mass spectrometry *(5)*.

The successful use of these methods based on Western blotting relies on the ability to visualize the total protein pattern present on the 2-DE gel, either to pinpoint the particular protein detected by reaction with a specific antibody or ligand or to allow selection of proteins for chemical characterization. A variety of methods have been described for the visualization of the total protein pattern following Western blotting. Some of these methods together with an indication of their relative sensitivities are given in **Table 1**. Fast green FC and Ponceau S are relatively insensitive stains, but can be easily removed from proteins after detection to allow for subsequent immunoprobing or chemical characterization. Amido black 10B is the most commonly used stain for the rapid and relatively sensitive visualization of proteins on nitrocellulose membranes. Coomassie brilliant blue R-250 gives a very high background on nitrocellulose, but gives excellent results with PVDF membranes. Blots stained with Coomassie brilliant blue R-250 or amido black 10B can be used for the chemical characterization of proteins, but they are not compatible with immunodetection methods. Instaview Nitrocellulose (BDH Merck, Poole, Dorset, UK) is a rapid and sensitive method of general protein staining with the advantage that the membrane can be rapidly destained without loss of immunoreactivity of the proteins, which can then be used in specific immunodetection protocols. More sensitive staining can be achieved by staining with india ink *(6)* or colloi-

dal gold particles *(7)*, but these procedures are more protracted (several hours to overnight). Recently, several reversible metal chelate stains of varying sensitivity have been developed *(8,9)*, which are compatible with both subsequent immunodetection protocols and methods for chemical characterization. Charged membranes present a particular problem, since the membrane surface can bind dyes as avidly as the separated proteins. For example, Immobilon-CD (Millipore, Bedford, MA) is a PVDF matrix that has been modified to carry a cationic charge on the surface and has been optimized for use in internal sequencing protocols. Proteins bind strongly to this membrane during blotting, and after proteolytic cleavage, the resulting peptide fragments can be released from the surface by mild conditions that disrupt the electrostatic interactions (e.g., changes in salt concentration or pH) *(10)*. Immobilon-CD Quick Stain (Millipore) is a negative dye that interacts solely with the membrane and, therefore, does not interfere with subsequent sequence analysis of the proteins. Nylon membranes are more difficult to stain, but this is possible using a colloidal iron procedure *(11)* or a method based on *in situ* biotinylation of the blotted proteins followed by visualization with peroxidase-conjugated avidin. In this chapter, methods are described for the detection of proteins on Western blot transfers with Ponceau S, Amido black 10B, Coomassie brilliant blue R-250, Immobilon-CD Quick Stain, Instaview Nitrocellulose, and india ink. Methods for protein detection using metal chelate stains are described in Chapter 37.

2. Materials

Prepare all solutions from analytical-grade reagents, and dissolve in deionized water.

1. Ponceau S staining solution (100 mL): 0.2% (w/v) Ponceau S in 10% (v/v) acetic acid. To prepare the staining solution, add 10 mL acetic acid to 80 mL deionized water. Add 0.2 g Ponceau S, and dissolve with gentle shaking. Make up to 100 mL. The solution can be stored at room temperature for up to 3 mo.
2. Ponceau S destaining solution: deionized water.
3. Amido black 10B staining solution: 0.1% (w/v) Amido black in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid. To prepare the staining solution, add 25 mL propan-2-ol and 10 mL acetic acid to 50 mL deionized water. Add 0.1 g Amido black, and dissolve with gentle shaking. Make up to 100 mL. The solution can be stored at room temperature for up to 3 mo.
4. Amido black destaining solution: 5% (v/v) propan-2-ol, 10% (v/v) acetic acid. To prepare the destaining solution, add 125 mL propan-2-ol and 50 mL acetic acid to 325 mL deionized water.
5. Coomassie brilliant blue R-250 staining solution: 0.2% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid. To prepare the staining solution, add 45 mL methanol and 10 mL acetic acid to 40 mL deionized

water. Add 0.2 g Coomassie brilliant blue R-250 and dissolve with gentle shaking. Make up to 100 mL. The solution can be stored at room temperature for up to 3 mo.

6. Coomassie destaining solution: 45% (v/v) methanol, 10% (v/v) acetic acid. To prepare the destaining solution, add 225 mL methanol and 50 mL acetic acid to 225 mL deionized water.

7. Immobilon-CD Quick Stain: To prepare a stock solution of the stain, add 60 mL deionized water to the bottle that contains the stain powder, and then heat at 37°C with gentle shaking until the reagent is completely dissolved (about 15 min). Store the stock staining solution in 8-mL aliquots at –20°C. The dry powder is stable when stored at 4°C (Millipore Corporation).

8. Immobilon-CD Quick stain developer: To prepare a stock solution of the developer, add 60 mL deionized water to the bottle that contains the developer powder, and then heat at 37°C with gentle shaking until the reagent is completely dissolved (about 15 min). Store the stock-developing solution in 8-mL aliquots at –20°C. The dry powder is stable when stored at 4°C.

9. Instaview Nitrocellulose staining kit (BDH Merck Ltd, Poole, Dorset, UK). The kit comprises three components: 50X stain concentrate, 100X enhancer concentrate, and 10X destain concentrate. These concentrates are stable when stored at room temperature.

10. India ink staining solution: 0.1% (w/v) india ink, 0.5% Tween 20, 1% (v/v) acetic acid. To prepare the staining solution, add 0.1 mL Pelikan Fount india ink (Pelikan AG, Hanover, Germany), 0.5 mL Tween 20, and 1 mL acetic acid to 90 mL deionized water. Stir for 60 min, filter, and then make up to 100 mL. Prepare fresh each time.

11. India ink destaining solution (500 mL): 154 mM sodium chloride. To prepare the destaining solution, dissolve 4.5 g sodium chloride and make up to 500 mL. Prepare fresh each time.

12. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$. To prepare the solution, dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, and 0.24 g KH$_2$PO$_4$ in 800 mL water. Adjust to pH 7.4 with HCl, and make up to 1 L.

13. Rocking platform.

14. Plastic boxes or glass dishes for staining and destaining blot membranes.

3. Methods

3.1. Ponceau S

1. Place the nitrocellulose or PVDF membrane (*see* **Note 1**) in the Ponceau S staining solution (*see* **Note 2**) and agitate for 30 min on the rocking platform.

2. Wash excess dye off the filter by rinsing in several changes of distilled water. An example of a membrane stained with Ponceau S is shown in **Fig. 1**.

3. The membrane can be completely destained by washing in PBS. After complete destaining, proteins can be detected by specific immunoprobing or subjected to chemical characterization (*see* **Note 3**).
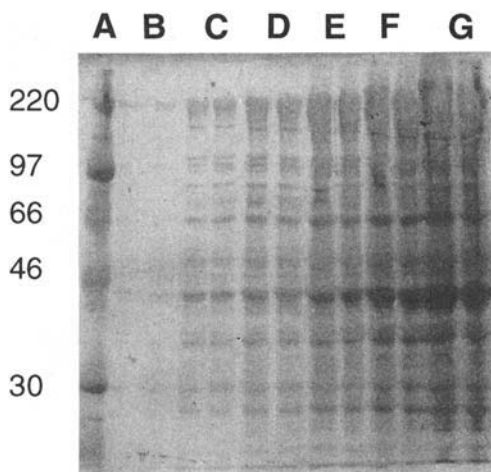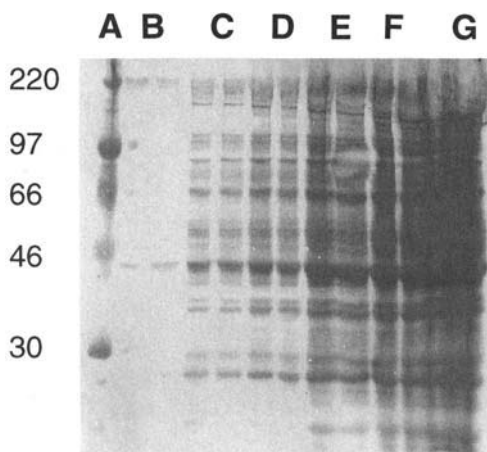
Fig. 1. Separation of human heart proteins by 10% T SDS-PAGE, electroblotted onto nitrocellulose and visualized by staining with Ponceau S. (A) Molecular-weight markers. (B–G) Human heart proteins. (B) 1 μg. (C) 5 μg. (D) 10 μg. (E) 25 μg. (F) 50 μg. (G) 100 μg.

3.2. Amido Black 10B

1. Place the nitrocellulose or PVDF membrane (*see* **Note 1**) in the Amido black staining solution (*see* **Note 2**), and agitate for 2 min on the rocking platform.
2. Place the membrane into destaining solution, and agitate for 10–15 min on the rocking platform (or until the background is pale). Destaining can be accelerated by using several changes of destaining solution. An example of a membrane stained with Amido black 10B is shown in **Fig. 2**.
3. After destaining, proteins can be subjected to chemical characterization (*see* **Note 3**). Blots stained with Amido black are not compatible with subsequent immunodetection methods.
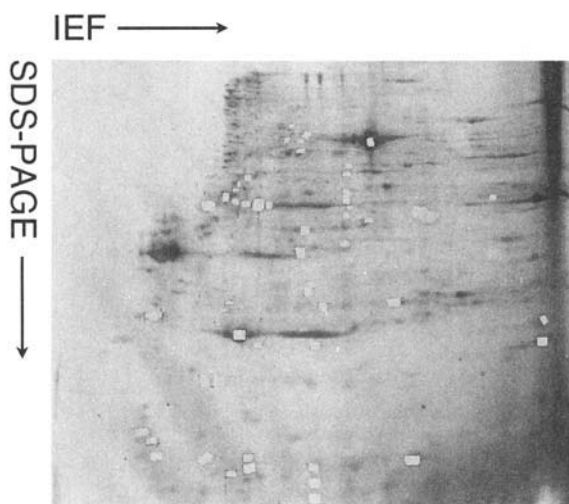
3.3. Coomassie Brilliant Blue R-250

1. Place the PVDF membrane (*see* **Note 1**) in the Coomassie brilliant blue staining solution (*see* **Note 2**), and agitate for 2 min on the rocking platform (*see* **Note 4**).
2. Place the membrane into destaining solution, and agitate for 10–15 min (or until the background is pale). Destaining can be accelerated by using several changes of destaining solution. Examples of membranes stained with Coomassie brilliant blue R-250 are shown in **Figs. 3** and **4**.
3. After destaining, proteins can be subjected to chemical characterization (*see* **Note 3**). Blots stained with Coomassie brilliant blue are not compatible with subsequent immunodetection methods.

Fig. 2. Separation of human heart proteins by 10% T SDS-PAGE, electroblotted onto nitrocellulose and visualized by staining with Amido black 10B. (A) Molecular-weight markers. (B–G) Human heart proteins. (B) 1 μg. (C) 5 μg. (D) 10 μg. (E) 25 μg. (F) 50 μg. (G) 100 μg.

3.4. Immobilon-CD Quick Stain

1. Prepare a working staining solution by the addition of 8 mL of stock stain solution to 92 mL deionized water (*see* **Note 2**) and warm to 50°C.
2. Prepare a working developing solution by the addition of 8 mL of stock developing solution to 92 mL deionized water (*see* **Note 2**) and warm to 50°C.
3. Rinse the Immobilon-CD blot membrane briefly in deionized water after transfer (*see* **Note 5**).
4. Place the membrane in the diluted staining solution, and agitate on the rocking platform for 5 min.
5. Place the membrane in the diluted developing solution, and agitate on the rocking platform for 3–5 min until the negatively stained protein zones are visible.
6. Rinse the membrane briefly in deionized water. An example a membrane negatively stained by the Immobilon-CD Quick Stain protocol is shown in **Fig. 5**.
7. After destaining, proteins can be subjected to chemical characterization (*see* **Note 6**). Immobilon-CD membranes are not compatible with immunodetection protocols.

3.5. Instaview Nitrocellulose

1. Prepare a working staining solution by the addition of 1 mL of 100X enhancer concentrate and 2 mL 50X stain concentrate to 97 mL deionized water (*see* **Note 2**) and mix well.
2. Place the nitrocellulose or PVDF membrane (*see* **Note 1**) to be stained in the staining solution, and agitate on the rocking platform for 2–5 min.
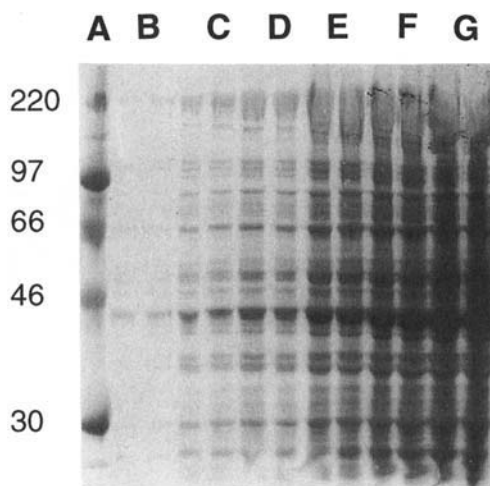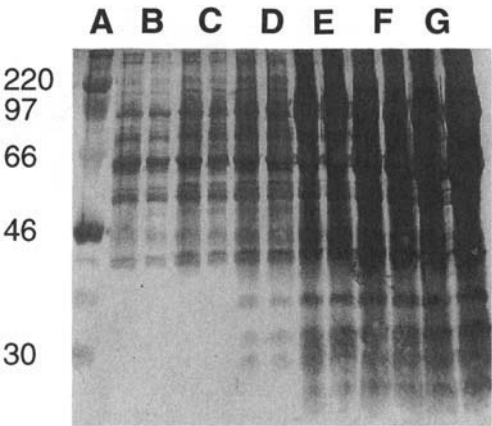
Fig. 3. Separation of human heart proteins by 10% T SDS-PAGE, electroblotted onto PVDF and visualized by staining with Coomassie brilliant blue R-250. (A) Molecular-weight markers. (B–G) Human heart proteins. (B) 1 μg. (C) 5 μg. (D) 10 μg. (E) 25 μg. (F) 50 μg. (G) 100 μg.

3. While the membrane is staining, prepare a working solution of the enhancing solution by addition of 1 mL 100X enhancer concentrate to 99 mL deionized water (*see* **Note 2**), and mix well.
4. Pour off the staining solution from the membrane, and replace with the working enhancing solution. Agitate on the rocking platform until a clear background is obtained (usually <5 min) (*see* **Note 7**). An example of a membrane stained with the Instaview Nitrocellulose protocol is shown in **Fig. 6**.
5. At this stage, the membrane may be dried and stored, or destained (*see* **step 6** and **Note 8**).
6. Prepare a working destaining solution by addition of 10 mL 10X destain concentrate to 90 mL deionized water (*see* **Note 2**).
7. Place the membrane in the destain solution and agitate until all the color has been removed (typically 5–10 min).

3.6. India Ink

1. Place the nitrocellulose or PVDF membrane (*see* **Note 1**) in the India ink staining solution (*see* **Note 2**), and agitate for 60 min on the rocking platform.
2. Place the membrane in destaining solution for 5 min (or until a pale background is obtained). An example of a membrane stained with india ink is shown in **Fig. 7**.
3. After destaining, proteins can be subjected to chemical characterization (*see* **Note 3**). Blots stained with India ink are not compatible with subsequent immunodetection methods.

Fig. 4. PVDF (FluoroTrans) Western electroblot transfer of 300 μg of human heart proteins separated by 2-DE and stained with Coomassie brilliant blue R-250. The white rectangular areas indicate protein spots which have been excised for chemical characterization.

4. Notes

1. If the PVDF membrane to be stained has been dried after electroblotting, then it must first be rewetted with methanol and then with deionized water prior to staining. A dry nitrocellulose membrane should be rewetted directly with deionized water prior to staining.

2. The volume of staining solution required depends on the size of the blotting membrane to be stained. A guide to the volume required is given in **Table 2**. The staining box or dish should have dimensions that are similar to the size of the membrane to minimize the volume of staining solution required. The solutions must completely cover the membrane.

3. Membranes should be washed well with several changes of deionized water, then placed on a filter paper, and allowed to dry. The dry membranes should be stored in sealed clean plastic bags until required for further analysis. The membranes can be stored in this state at room temperature for extended periods without any apparent adverse effects on subsequent specific immunodetection or chemical characterization.

4. Nitrocellulose membranes should not be stained with Coomassie brilliant blue R-250 as this results in a very high background.

5. Immobilon-CD membranes must not be allowed to dry between the blotting and staining protocols.

6. Stained Immobilon-CD membranes should be stored wet at –20°C for subsequent chemical characterization. Excess stain can be removed from the protein
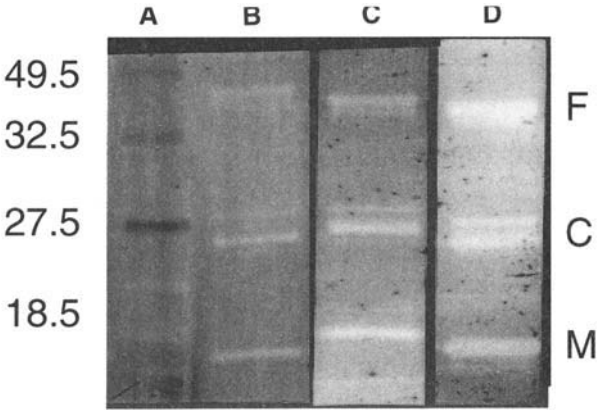
Fig. 5. Standard proteins separated by SDS-PAGE, electroblotted onto Immobilon-CD membranes and visualized with the Immobilon-CD Quick Stain kit. (A) Prestained molecular-weight markers. (B–D) Standard proteins (F, bovine fetuin; C, bovine β-casein; M, horse heart myoglobin). (B) 5 pmol. (C) 15 pmol. (D) 50 pmol loadings of each standard protein.
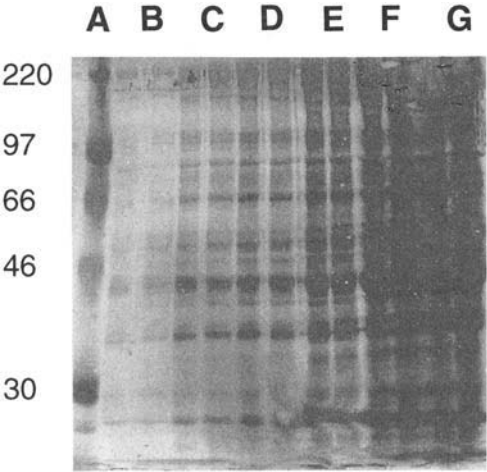
Fig. 6. Separation of human heart proteins by 10% T SDS-PAGE, electroblotted onto nitrocellulose and visualized by staining with Instaview Nitrocellulose. (A) Molecular-weight markers. (B–G) Human heart proteins. (B) 1 μg. (C) 5 μg. (D) 10 μg. (E) 25 μg. (F) 50 μg. (G) 100 μg.

zones prior to analysis by gently shaking the excised membrane piece in 1 mL of acetone containing 0.1% (v/v) cyclohexamine for 1–2 min. The membrane piece should then be air-dried.

Fig. 7. Separation of human heart proteins by 10% T SDS-PAGE, electroblotted onto nitrocellulose and visualized by staining with india ink. (A) Molecular-weight markers. (B–G) Human heart proteins. (B) 1 μg. (C) 5 μg. (D) 10 μg. (E) 25 μg. (F) 50 μg. (G) 100 μg.

Table 2
Approximate Volume of Staining Solution Required
for Various Sizes of Blotting Membranes

Blot size, cm	Volume of stain, mL
8 × 10	25
15 × 15	50
20 × 20	100
26 × 26	175

7. With PVDF membranes, a blue background staining may still be observed.
8. The destaining step completely destains the protein bands and the background of the blot, allowing the subsequent use of specific methods of immunoprobing the membrane using normal protocols.

Acknowledgments

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37

Protein Detection
Using Reversible Metal Chelate Stains

Wayne F. Patton, Mark J. Lim, and David Shepro

1. Introduction

Electrophoretically separated proteins may be visualized using organic dyes, such as Ponceau red, Amido black, fast green, or most commonly Coomassie Brilliant Blue *(1,2)*. Alternatively, sensitive detection methods have been devised using metal ions and colloids of gold, silver, copper, carbon, or iron *(3–12)*. Metal chelates form a third class of stains, consisting of organometallic complexes that bind avidly to proteins resolved in polyacrylamide gels or immobilized on solid-phase supports *(13)*. The metal chelate staining procedures are simple, requiring reagents that are easily prepared, are stable at room temperature, and can be reused several times without loss of sensitivity. The staining procedures are relatively inexpensive, since they do not utilize precious metals, such as gold or silver. Like Ponceau red stain, the metal chelate stains are readily reversible. Complexes form at acidic pH and elute on increasing the pH to 7.0–10.0. Metal chelates can be used to detect proteins on nitrocellulose, PVDF, and nylon membranes as well as in polyacrylamide gels. The metal complexes do not modify proteins, and are compatible with immunoblotting, lectin blotting, mass spectrometry, and Edman-based protein sequencing *(13–17)*. Metal chelate stains are suitable for routine protein measurement in solid-phase assays owing to the quantitative stoichiometry of complex formation with proteins and peptides *(15,16)*. Such solid-phase protein assays are more sensitive and resistant to chemical interference than their solution-based counterparts *(15)*.

Metal chelate stains display a number of common structural features (**Fig. 1**). They possess a domain that binds to metal ions, preferably containing side
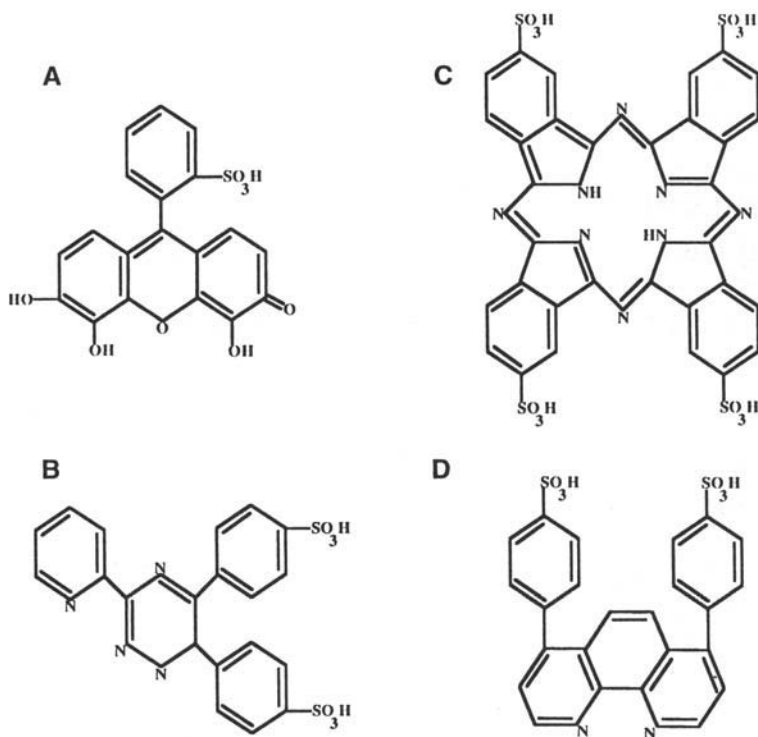
Fig. 1. Chemical structures of four representative metal chelate stains. **(A)** Pyrogallol red (binds molybdenum) **(B)** Ferrozine (binds iron) **(C)** Phthalocyanine tetrasulfonate (binds copper). **(D)** Bathophenanthroline disulfonate (binds europium). The molecules contain aromatic rings, metal binding sites, and sulfonic acid side chains.

chains that are carboxyl, hydroxyl, ketone, or pyridine groups. They contain a domain responsible for detection of the metal ion, preferably consisting of five- or six-member monocyclic or polycyclic, aromatic, or heteroaromatic ring structures, a necessity for chromophores. The aromatic or heteroaromatic functionalities may also enhance association with proteins through hydrophobic interactions. Finally, they contain a domain that reversibly binds to proteins, consisting of one or more anionic groups, preferably sulfonate residues. This is necessary for selective electrostatic interaction with α- and ε-amino side chains in proteins at acidic pH (pH 2.0–4.0).

A variety of metal ions and organic chelating agents may be combined to form metal chelate stains, but only a few have been utilized for the specific detection of iron-containing proteins, such as cytochrome-*c*, transferrin, ferritin, and lactoferrin in polyacrylamide gels *(18)*. In this situation, the organometallic complex only forms when the chelate interacts with the metal ion

bound within the protein itself. Copper phthalocyanine 4, 4',4", 4''' tetrasulfonic acid has been shown to stain total protein in electrophoresis gels and on nitrocellulose membranes *(19)*. Recently, we demonstrated the use of Ferrene S/ferrous, Ferrozine/ferrous, ferrocyanide/ferric, and Pyrogallol red-molybdate complexes for colorimetric detection of electrophoretically separated proteins immobilized on membranes *(13–16)*.

In 1978, a pink bathophenanthroline disulfonate-ferrous complex was reported as a nonspecific protein stain for polyacrylamide gel electrophoresis *(20)*. The stain is rather insensitive and was later modified by substituting [^{59}Fe] into the complex in order to detect proteins by autoradiography *(21)*. Though increasing sensitivity substantially, the hazards associated with working with radioactivity and the burden of license application for an infrequently used radioisotope have precluded routine utilization of bathophenanthroline disulfonate-[^{59}Fe] as a general protein stain. Recently, we substituted europium into the bathophenanthroline disulfonate complex to form a luminescent metal chelate stain *(17)*. The bathophenanthroline disulfonate-europium complex is useful for the reversible detection of low-nanogram quantities of proteins and nucleic acids immobilized on membrane supports and in polyacrylamide gels.

Measuring light emission is intrinsically more sensitive than measuring light absorbance, since the later is limited by the molar extinction coefficient of the colored complex *(22)*. Thus, luminescent protein detection systems utilizing chelates complexed to lanthanide metal ions, such as europium or terbium, should offer greater sensitivity than their colorimetric counterparts. The organic component of the complex absorbs light and transfers the energy to the lanthanide ion, which subsequently emits light at longer wavelength. Regardless of the chelate, terbium and europium each generate two narrow emission bands (λ_{max} = 491 and 545 nm for terbium, λ_{max} = 590 and 615 nm for europium) corresponding to D to F electronic transitions. Thus, terbium complexes emit green light, and europium complexes emit red light. Although luminescent emission is a characteristic of the lanthanide ion, absorption of the excitation light depends on the organic chromophore portion of the metal complex. Linear aliphatic chelates, such as EDTA and EGTA, maximally absorb short wavelength UV light (200–230 nm), whereas aromatic or heteroaromatic chelates maximally absorb long wavelength UV light (325–375 nm) *(17)*. Lanthanide ions emit at least 200 times more light when complexed to organic chelates *(17)*. A potential drawback to detection of proteins using luminescent compared to colorimetric metal chelate stains is the requirement for ancillary equipment, such as a UV light box, band pass filters, and camera. A potential advantage is that the lanthanide metal chelates are only visible on illumination with a UV light source, thus obviating the need to destain before utilizing colorimetric immunodetection procedures, such as the commonly employed

bromochloroindoyl phosphate/nitroblue tetrazolium (BCIP/NBT) method of detecting alkaline phosphatase-conjugated secondary antibodies *(23)*.

Procedures for the detection of electrophoretically separated proteins utilizing colorimetric and luminescent metal chelate stains are presented in this chapter. Methods for the elution of the metal chelate stains are also presented. The procedures are applicable for detection of proteins or peptides in polyacrylamide gels or on nitrocellulose, polyvinyl difluoride, or nylon membranes. Owing to the electrostatic mechanism of the protein visualization methods, metal chelate stains are unsuitable for detection of proteins and peptides immobilized on cationic membranes.

2. Materials

2.1. Colorimetric Detection of Proteins on Nitrocellulose, PVDF, or Nylon Membranes

1. Block buffer: 0. 1% polyvinylpyrrolidone-40 (PVP-40) in 2% glacial acetic acid.
2. Ferrozine/ferrous stain (stable for at least 6 mo at room temperature): 0.75 mM 3(2-pyridyl)-5,6-*bis*(4-phenyl sulfonic acid)-1,2,4-triazine disodium salt (Ferrozine), 30 mM ferric chloride, 15 mM thioglycolic acid in 2% glacial acetic acid. Alternatively, commercially prepared stain solutions of Ferrozine-ferrous (Rev-Pro stain kit; ESA, Chelmsford, MA) or Pyrogallol red-molybdenum (Microprotein-PR kit; Sigma Chemical Company, St. Louis, MO) can be used.
3. 2% Glacial acetic acid.
4. Ferrocyanide/ferric stain (stable for at least 6 mo at room temperature): 100 mM sodium acetate, pH 4.0, 100 mM potassium ferrocyanide, 60 mM ferric chloride.
5. Ferrozine/ferrous elution solution: 50 mM Tris-HCl, pH 8.8, 200 mM NaCl, 20 mM EDTA.
6. Destain: 200 mM sodium carbonate, 100 mM EDTA, pH 9.6.

2.2. Luminescent Detection of Electroblotted Proteins

1. Formate buffer: 100 mM formic acid, pH 3.7, 100 mM sodium chloride.
2. Bathophenanthrohne/europium blot stain (stable for at least 6 mo at room temperature): 1.5 mM bathophenanthroline disulfonic acid disodium salt, 0.5 mM europium chloride, and 0.2 mM EDTA (added from 1000X stock, pH 7.0).
3. Ferrozine/ferrous stain enhanced with the ferrocyanide/ferric elution: 200 mM sodium carbonate, 100 mM EDTA, pH 9.6.

2.3. Luminescent Detection of Proteins in SDS-Polyacrylamide Gels

1. 25% (w/v) Trichloroacetic acid.
2. 30% Methanol.
3. Bathophenanthroline/europium gel stain (stable for at least 6 mo at room temperature): 0.15 mM bathophenanthroline disulfonic acid disodium salt, 0.05 mM europium chloride, and 0.02 mM EDTA (added from 1000X stock, pH 7.0), in 30% methanol.

4. Bathophenanthroline/europium elution I: 200 m*M* sodium carbonate, 100 m*M* EDTA, pH 9.6.

5. Bathophenanthroline/europium elution II: 200 m*M* sodium carbonate, 100 m*M* EDTA, pH 9.6, in 30% methanol.

3. Methods

3.1. Colorimetric Detection of Proteins on Nitrocellulose, PVDF, or Nylon Membranes

The colorimetric metal chelate stains allow rapid visualization of proteins on solid-phase supports with detection sensitivities that are comparable to Coomassie brilliant blue staining *(13–16)*. Detection sensitivity of the Ferrozine/ferrous stain can be enhanced to a level comparable to silver staining by further incubating membranes in ferrocyanide/ferric stain *(13,15)*. The colorimetric metal chelate stains are fully reversible and compatible with Edman-based protein sequencing, lectin blotting, mass spectrometry and immunoblotting *(13–16)*.

1. After electroblotting (*see* Chapter 35), completely immerse membranes in block buffer for 10 min. Perform blocking and staining steps on a rotary shaker (50 rpm).
2. Thoroughly immerse membranes in Ferrozine/ferrous stain for 10–15 min until purple bands or spots appear.
3. Remove unbound dye by several brief rinses (five to seven) in 2% glacial acetic acid until the membrane background appears white. Shaking can be performed manually using wash volumes roughly two times greater than in the blocking and staining steps.
4. If increased sensitivity is desired, the blot can be double-stained by subsequently incubating in the ferrocyanide/ferric stain for 10–15 minutes (*see* **Notes 1–3**).
5. Remove unbound dye by several brief washes (five to seven) in 100 m*M* sodium acetate, pH 4.0 (*see* **Note 4**). Shaking can be performed manually using wash volumes roughly two times greater than in the blocking and staining steps.
6. Visualize stained proteins by eye, and quantify transmissively using a CCD camera (*see* Chapters 39 and 40).

3.2. Luminescent Detection of Proteins on Nitrocellulose Membranes

Luminescent metal chelate stains offer the same advantages as colorimetric stains, but with the additional benefits of a 500-fold linear dynamic range, detection sensitivity of <1 ng/mm^2 of membrane surface, and capability of detecting nucleic acids as well as proteins *(17)*.

1. Following electroblotting (Chapter 35), wash membranes 2 × 30 min with formate buffer followed by four times for 30 min with deionized water (*see* **Note 5**). All washing and staining steps are performed on a rotary shaker (50 rpm).
2. Completely immerse membranes in the bathophenanthroline/europium blot stain for 15 min.

Isoelectric point (pI)
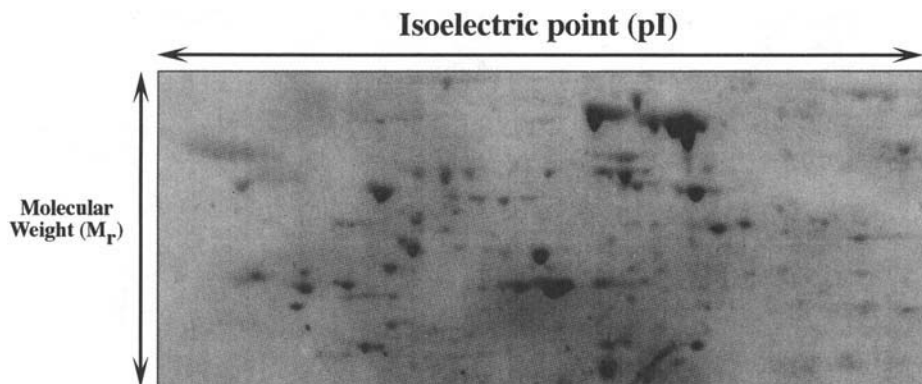
Fig. 2. Region of an electroblot of human omentum microvascular endothelial cells separated by 2-D gel electrophoresis. Proteins were visualized utilizing batho-phenanthroline /europium stain as described in **Subheading 3.2.** The blot was illuminated with 302 nm UV light and red luminescence detected using a CCD camera as described in Chapter 39. Since most image analysis software programs assume that the proteins are dark and the background is white, the gray scale has been inverted in the image.

3. Remove unbound dye by washing four to six times for 1 min in deionized water (*see* **Note 6**).
4. Dry membranes at room temperature or in a 37°C drying oven (for quicker results) following the washing steps (*see* **Note 7**).

3.3. Luminescent Detection of Proteins in SDS-Polyacrylamide Gels

Luminescent detection of proteins in polyacrylamide gels with batho-phenanthroline/europium is as simple as Coomassie brilliant blue staining, but provides detection sensitivity comparable to silver staining *(17)*.

1. Following electrophoresis, fix gels for 20 min in 25% (w/v) trichloroacetic acid. All steps are performed with gentle agitation on a rotary shaker (50 rpm).
2. Wash gels 3 × 20 min in 30% methanol (*see* **Note 8**).
3. Stain gels for 1–2 h in bathophenanthroline/europium gel stain (*see* **Note 9**).
4. Destain gels by washing three times for 5 min in 30% methanol (*see* **Note 9**).
5. Visualize stained proteins by transmissive UV illumination (*see* Chapter 39).

3.4. Elution of Metal Chelate Stains

The Ferrozine/ferrous stain can be eluted by immersing the blots in 50 m*M* Tris-HCl, pH 8.8, 200 m*M* NaCl, and 20 m*M* EDTA for 15 min on a rotary shaker (50 rpm). Alternatively, phosphate-buffered saline, pH 7.0, can be employed. Blots treated with the Ferrozine/ferrous stain followed by enhancement with the ferrocyanide/ferric stain require harsher elution conditions. This

stain is eluted by incubation in 200 m*M* sodium carbonate and 100 m*M* EDTA, pH 9.6, for 10 min. Since the bathophenanthroline/europium stain can only be observed upon UV illumination, it does not require elution if subsequent colorimetric detection procedures are to be performed. If concerned about interference with subsequent procedures, however, the stain can be eluted from blots by incubation in 100 m*M* sodium carbonate and 100 m*M* EDTA, pH 9.6, for 15 min. For gels, the elution buffer should be prepared in 30% methanol, and incubation times should be extended to 1–2 h (depending on gel thickness).

4. Notes

1. Although enhancement of the Ferrozine/ferrous stain with ferrocyanide/ferric stain substantially increases detection sensitivity, the double stain is also more difficult to elute than the Ferrozine/ferrous stain alone (*see* **Subheading 3.4.** for methods of stain reversal).
2. The ferrocyanide/ferric stain may form a precipitate after long-term storage. The precipitate is easily resuspended by vigorous shaking or sonication.
3. If a dried nitrocellulose membrane is incubated in the ferrocyanide/ferric stain, a patchy background may result that is difficult to destain. Dry blots should be rehydrated briefly in deionized water prior to incubation in the ferrocyanide/ferric stain.
4. The number of washes necessary to remove background staining may vary slightly. The ferrocyanide/ferric stain may initially remain bound to the nitrocellulose membrane, but the membrane background will become white with sufficient washing.
5. Since formate ions may chelate europium ions, the washes with deionized water are crucial for complete removal of the formate buffer. Otherwise, the staining solution may be inactivated. Care should be taken to aspirate off all the solution between washes.
6. Removal of unbound bathophenathroline/europium stain cannot be visually monitored as with the colorimetric stains. We have found that washing four times for 1 min is effective. However, the number and duration of washes may vary from case to case (depending on the size of the membrane and the volume of each wash). Therefore, optimal washing should be determined empirically for each application.
7. After staining, wet membranes should not be touched, since residue found on latex laboratory gloves may destroy the stain. Once dry, membranes can be handled freely. Since water is known to quench europium luminescence, drying the membrane also serves to enhance the signal (about twofold).
8. Trichloroacetic acid chelates europium ions and will destroy the staining solution. Therefore, the washes are extremely important to the success of the stain. Incubation times may vary depending on the volume of washing solution used and the gel thickness and size. This should be one of the first steps evaluated when troubleshooting.
9. Staining and destaining steps may vary depending on gel thickness and size. Our procedures have been optimized using 4–15% SDS polyacrylamide PhastGels (Pharmacia LKB Biotechnology, Piscataway, NJ).

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38

Glycoprotein Detection of 2-D Separated Proteins

Nicolle H. Packer, Malcolm S. Ball, and Peter L. Devine

1. Introduction

The profile of the two-dimensional (2-D) separation of the protein comple-
ment (proteome) of eukaryotic cells and tissues typically contains obvious
"trains" of spots that differ in p*I* and/or apparent molecular mass. These are
usually isoforms of the same protein and result from a variety of posttransla-
tional modifications. There is growing evidence that alterations to the
glycosylation of a protein can be correlated with developmental and pathologi-
cal changes; these changes can be visualized on the 2-D separation by alter-
ations in the pattern of these glycoprotein isoforms.

The initial step, once a 2-D separation has been achieved, is to identify which
spots are the glycoproteins of interest, so that further characterization can pro-
ceed. Various methods have been developed for the detection of glycoproteins
on 2-D gels *(1)* and blots by color and lectin analysis, and these can be carried
out at the analytical level. The actual level of detection of course depends on
the extent of glycosylation of the protein, since the reagents react only with the
carbohydrate moiety. We have chosen to describe here the stains that we have
found to be the most useful for visualizing, both on gels and blots, the glyco-
proteins separated by 2-D electrophoresis. A protocol for analyzing the
monosaccharide composition of these glycoprotein spots is also described.

1. Periodic acid/Schiff staining is a generally useful technique for locating glyco-
   proteins on gels and nitrocellulose blots, though the sensitivity may not be suffi-
   cient for some applications. Realistically, 1–10 µg of a highly glycosylated
   protein can be detected. Periodic acid oxidizes vicinal diols of glycosyl residues
   to dialdehydes. The aldehydes are then allowed to react with fuchsin (Schiff's
   reagent) to form a Schiff base. Glycoproteins stain pink with fuchsin on a clear
   background.

2. Digoxigenin (DIG)/AntiDIG alkaline phosphatase (AP) labeling is an extension of the periodic acid–Schiff method above, although the sensitivity is much greater (realistically, depending on the degree of glycosylation, about 0.1 µg glycoprotein). Glycoproteins can be detected on dot blots or after Western transfer to membranes, such as nitrocellulose or PVDF. Vicinal (adjacent) hydroxyl groups in sugars of glycoconjugates are oxidized to aldehyde groups by mild periodate treatment. The spacer-linked steroid hapten digoxigenin (DIG) is then covalently attached to these aldehydes via a hydrazide group. DIG-labeled glycoconjugates are subsequently detected in an enzyme immunoassay using a DIG-specific antibody conjugated with AP. DIG glycan detection is known to label almost all known N- and O-linked glycans, including GPI anchors.

3. Lectins are carbohydrate binding proteins, which are particularly useful in glycoprotein and carbohydrate analysis, since they can be conjugated to a variety of enzymes or haptens for use in sensitive detection systems. Their specificity can be used to probe for specific structures in the glycoconjugates. Lectins are usually classified on the basis of the monosaccharides with which they interact best, but it is important to note that complex glycoconjugates are generally found to be much better ligands. In addition, the position of a particular monosaccharide in a glycan chain (i.e., to what it is attached) will affect lectin binding, so results obtained in lectin binding studies should be treated with caution. For example, (a) the wheat germ agglutin (WGA) is inhibited most strongly by dimeric GlcNAc, but in glycoproteins this lectin also reacts very strongly with sialic acid and peptide-linked GalNAc; (b) the peanut agglutin binds to Gal1,3GalNAc, but does not react when this structure is sialylated.

4. The monosaccharide composition of a glycoprotein is a useful start to full characterization. This is obtained by hydrolysis of the separated glycoprotein spots, which have been electroblotted to PVDF, followed by monosaccharide analysis using high-pressure anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) *(2)*.

In all cases, it is useful to include a glycosylated protein, such as transferrin or ovalbumin in the marker lane as a control.

2. Materials

2.1. Reagents for Periodic Acid/Schiff Staining

1. Solution A: 1.0% (v/v) periodic acid in 3% acetic acid. Periodic acid is corrosive and volatile—handle with caution. Be aware of the concentration of periodic acid in the solution that is being diluted, since periodic acid is only about 50% out of the reagent bottle.
2. Solution B: 0.1% (w/v) sodium metabisulfite in 10 m*M* HCl.
3. Schiff's reagent: A commercial reagent from Sigma (St. Louis, MO) may be used, or better staining can often be achieved by making fresh reagent:
   a. Dissolve 1 g basic fuchsin in 200 mL of boiling distilled water, stir for 5 min, and cool to 50°C.

b. Filter and add 20 mL 1 *M* HCl to filtrate.

c. Cool to 25°C, add 1 g potassium metabisulfite, and leave to stand in the dark for 24 h.

d. Add 2 g activated charcoal, shake for 1 min, and filter. Store at room temperature in the dark.

Schiff's reagent is corrosive and slightly toxic, and a very dilute solution will stain anything with oxidized carbohydrates a pink-purple color—wear gloves and protective clothing when using this solution and washing it out of the gel/blot.

4. Solution C: 50% (v/v) ethanol.
5. Solution D: 0.5% (w/v) sodium metabisulfite in 10 m*M* HCl.
6. Solution E: 7.5% (v/v) acetic acid /5% (v/v) methanol in distilled water.
   Solutions A, B, and D should be made up freshly.

2.2. Reagents for Digoxigenin/Antidigoxigenin, AP Labeling

1. Buffer A (TBS): 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
2. Buffer B: 100 m*M* sodium acetate pH 5.5.
3. Buffer C: 100 m*M* Tris-HCl, pH 8.0, 50 m*M* MgCl$_2$, 100 m*M* NaCl.
4. Buffer D: 250 m*M* Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, bromophenol blue as tracking dye.
5. Buffer E: 50 m*M* potassium phosphate, 150 m*M* NaCl, pH 6.5 (PBS).
6. Blocking reagent: A fraction of milk proteins that are low in glycoproteins. 0.5 g is dissolved in 100 mL of buffer A. The solution should be heated to 60°C with stirring; the solution will remain turbid. Allow the solution to cool before immersing the membrane.
7. DIG glycan detection kit (Boehringer-Mannheim, Mannheim, Germany) containing:
   a. Solution 1: 10 m*M* sodium metaperiodate in buffer B.
   b. Solution 2: 3.3 mg/mL sodium metabisulfite.
   c. DIG-succinyl-ε-amidocaproic acid hydrazide.
   d. Anti-DIG-AP: Polyclonal sheep antidigoxigenin Fab fragments, conjugated with AP (750 U/mL).
   e. Solution 3: 75 mg/mL 4-nitroblue tetrazolium chloride dissolved in 70% (v/v) dimethylformamide.
   f. Solution 4: 50 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate dissolved in dimethylformamide.

Make sure that solutions 3 and 4 are still good, since after a few weeks, the solutions may begin to precipitate, thus reducing the staining efficiency. Store these solutions (3 and 4) in the dark.

2.3. Reagents for DIG-Labeled Lectin Staining

1. 1% KOH.
2. Blocking reagent (Boehringer-Mannheim): A fraction of milk proteins that are low in glycoproteins; 0.5 g is dissolved in 100 mL of TBS. The solution should be heated to 60°C with stirring. The solution will remain turbid. Allow the solution to cool before immersing the membrane. Other blockers, such as skim milk powder, gelatin, or BSA, may lead to high background.

3. TBS: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
4. TBS-Tween: TBS + 0.05% Tween-20.
5. Divalent cation stock solution: 0.1 *M* CaCl$_2$, 0.1 *M* MgCl$_2$, 0.1 *M* MnCl$_2$.
6. DIG-labeled lectins (Boehringer Mannheim): SNA, MAA, PNA, DSA.
7. Part of DIG glycan detection kit (Boehringer-Mannheim) comprising:
   a. Anti-DIG-AP: Polyclonal sheep anti-DIG Fab fragments, conjugated with AP (750 U/mL).
   b. Buffer C: 100 m*M* Tris-HCl, 50 m*M* MgCl$_2$, 100 m*M* NaCl.
   c. Solution 3: 75 mg/mL 4-nitroblue tetrazolium chloride dissolved in 70% (v/v) dimethylformamide.
   d. Solution 4: 50 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate dissolved in dimethylformamide.
   Store these solutions (3 and 4) in the dark.

2.4. Reagents for HPAEC Analysis of Monosaccharide Composition

2.4.1. Chemicals

1. Methanol.
2. 0.1*M* TFA.
3. 2 *M* TFA.
4. Standard sugars:
   a. 0.1 μmol/mL lactobionic acid.
   b. 0.1 μmol/mL 2-deoxyglucose.
   c. 0.1 μmol/mL *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid.
   d. 0.1 μmol/mL mixture of fucose, 2-deoxyglucose, galactosamine, glucosamine, galactose, glucose, and mannose.
   Sugars should be dried thoroughly over phosphorus pentoxide in a desiccator before weighing.

2.4.2. Instrumentation

Metal-free HPLC system with DIONEX CarboPac PA1 PA10 column , 4 mm × 25 cm and pulsed amperometric detector (HPAEC-PAD).

3. Methods

3.1. Periodic Acid/Schiff Staining

This method is essentially as described in Thornton et al. *(3)* for glycoproteins transferred to nitrocellulose membranes (*see* Chapter 35). A modification of the method for PVDF membranes is described in **ref. 4**.

3.1.1. Gel Staining (see **Note 4**)

1. Soak the gel in solution C for 30 min (*see* **Note 1**).
2. Wash in distilled water for 10 min. The gel must have all of the ethanol removed from the gel, so make sure that the gel is immersed in the water properly. If necessary, wash a second time to ensure the removal of the ethanol.

3. Incubate in solution A for 30 min. Beware of the fumes from the acid. From this point onward, the gel should be placed in the fume hood.
4. Wash in distilled water for at least 6 × 5 min or 5 × 5 min and 1x overnight.
5. Wash in solution B for 2 × 10 min. At this stage make up 100 mL solution B and do 2 × 30 mL washes. Save the final 40 mL for **step 7**.
6. Incubate in Schiff's reagent for 1 h in the dark. It is essential after adding the Schiff's reagent that the gel is kept in the dark. Any light will stop the color from developing.
7. Incubate in solution B for 1 h in the dark.
8. Wash several times in solution D for a total of at least 2 h, and leave as long as overnight to ensure good color detection (*see* **Note 2** and **3**).
9. Store the gel in solution E.

3.1.2. Membrane Staining (see **Note 5**)

1. Wash the membrane in distilled water for 5 min (*see* **Note 6**).
2. Incubate in solution A for 30 min.
3. Wash in distilled water for 2 × 5 min.
4. Wash in solution B for 2 × 5 min.
5. Incubate for 15 min in Schiff's reagent (*see* **Note 2**).
6. Wash in solution B for 2 × 5 min.
7. Air-dry membrane.

3.2. DIG/Anti-DIG, AP Labeling

This staining procedure can only be used on membranes (*see* Chapter 35). However, the proteins can be prelabeled in solution before electrophoresis or labeled on the membrane after blotting (*see* **Notes 8** and **9**). In both cases, the color development is the same. Nitrocellulose membranes can be used, but some background staining can occur with postlabeling. In preference, the proteins should be blotted onto PVDF.

The reagents for this method are provided in the Boehringer Mannheim DIG Glycan Detection Kit, and the methods described are essentially taken from that kit. A similar kit based on biotin/streptavidin binding instead of the digoxenin/antidigoxenin interaction is marketed by Bio-Rad (Hercules, CA) as the Immun-Blot® Glycoprotein Detection Kit.

3.2.1. Prelabeling (see **Note 8**)

1. Dilute protein solution 1:1 to 20 µL with buffer B (*see* **Note 7**).
2. Add 10 µL solution 1, and incubate for 20 min in the dark at room temperature.
3. Add 10 µL solution 2, and leave for 5 min. The addition of the sodium bisulfite destroys the excess periodate.
4. Add 5 µL DIG-succinyl-ε-amidocaproic acid hydrazide, mix, and incubate at room temperature for 1 h. Sensitivity may be increased by increasing the incubation time to several hours.

5. Add 15 μL buffer D, and heat the mixture to 100°C for 5 min to stop the labeling.
6. Separate the labeled glycoproteins by SDS-PAGE, and blot to membrane (*see* **Note 10**) that is now ready for staining reaction *(see below)*.

3.2.2. Postlabeling (see **Note 9**)

1. Wash the membrane for 10 min in 50 mL buffer E (PBS) (*see* **Note 12**).
2. Incubate the membrane in 20 mL solution 1 for 20 min at room temperature. For low amounts of oligosaccharide, it may be necessary to increase the amount of sodium metaperiodate in the solution. Increasing the concentration up to 200 m*M* increases the final staining.
3. Wash in buffer E, 3 × 10 min.
4. Incubate the membrane in 5 mL buffer B containing 1 mL DIG-succinyl-ε-amidocaproic acid hydrazide for 1 h at room temperature. For low amounts of glycoproteins, greater sensitivity can be obtained by increasing the concentration of DIG-succinyl-ε-amidocaproic acid hydrazide. However, no further benefit is gained by raising the concentration >3 mL in 5 mL (*see* **Note 11**).
5. Wash for 3 × 10 min in buffer A. TBS may now be used to wash the membrane, since the DIG labeling has taken place.

3.2.3. Staining Reaction

1. Incubate the membrane for at least 30 min in the blocking reagent (*see* **Note 10**). The membrane can be stored for several days at 4°C at this stage, and in fact, a lower background staining can be achieved by allowing the filter membrane to wash in the solution at 4°C overnight (shaking is not necessary) and then for 30 min at room temperature with shaking.
2. Wash for 3 × 10 min in buffer A.
3. Incubate the membrane with 10 mL buffer A containing 10 μL anti-DIG-AP at room temperature for 1 h. Sensitivity can be increased by increasing the amount of anti-DIG in the solution by a factor of 2 (although any more has no appreciable effect) or by increasing the incubation time to several hours.
4. Wash for 3 × 10 min with buffer A.
5. Immerse the membrane without shaking into 10 mL of buffer C containing 37.5 μL solution 4 and 50 μL solution 3 (mix just before use). If there is a lot of sugar present in the bands, the color reaction can take only a few minutes. However, if there is little material, the reaction could take several hours or overnight. The reaction is best done in the dark, since light can cause nonspecific staining of the membrane. If solutions 3 and 4 are not fresh, then the reaction can take several hours and cause a lot of background staining. The reaction can be sped up by doubling the amounts of solutions 3 and 4 added to buffer C.
6. Wash the membrane several times with Milli-Q water, and allow to air-dry. The membrane is best stored in foil to reduce fading of the bands once the reaction is stopped.

3.3. DIG-Labeled Lectin Staining

A wide range of lectins are commercially available as free lectin or conjugates of peroxidase, biotin, DIG, FITC, alcohol dehydrogenase, colloidal gold,

or solid supports, such as agarose. A list of commonly used commercially available lectins is shown in **Table 1**. Peroxidase- or alkaline phosphatase-labeled lectins can be detected directly. Alternatively, lectins can be detected using anti-DIG peroxidase (if DIG-labeled) or streptavidin peroxidase (if biotin-labeled), followed by an insoluble substrate. Sensitivity is generally increased with these indirect methods. The detection can be carried out with blots on nitrocellulose or PVDF (*see* **Note 15**).

1. Fix membrane for 5 min with 1% KOH.
2. Rinse for 1 min with distilled water.
3. Block unbound sites with a 1-h incubation at room temperature with blocking reagent.
4. Rinse away blocking reagent with 3 × 1 min washes with TBS-Tween.
5. Add DIG-labeled lectins diluted in TBS-Tween containing 0.1 m*M* CaCl$_2$, 0.1 m*M* MgCl$_2$, 0.1 m*M* MnCl$_2$. Leave overnight at 4°C (*see* **Notes 13** and **14**).
6. Remove unbound lectin by washing with 6 × 5 min in TBS-Tween.
7. Incubate nitrocellulose with anti-DIG AP 10 μL diluted in 10 mL TBS for 1 h room temperature.
8. Repeat washing **step 6**.
9. Immerse the membrane without shaking into 10 mL of buffer C containing 37.5 μL solution 4 and 50 μL solution 3 (mix just before use).
10. Stop reaction when gray to black spots are seen (a few minutes to overnight) by rinsing with water.

3.4. HPAEC Analysis of Monosaccharide Composition (see Note 16)

This technique requires about 100 pmol (5 μg) of highly glycosylated protein. Thus, it is only possible with the most abundant glycoproteins separated by preparative 2-D electrophoresis.

1. Excise spots that have been visualized by Coomassie blue or amido black on PVDF membranes and place into screw-capped Eppendorf tubes (*see* **Note 18**).
2. Wet membrane with methanol. If the membrane is not properly wetted, it will float and the hydrolysis will not be complete.

3.4.1. Analysis of Sialic Acids

1. Add 100 μL 0.1 *M* TFA to the wetted membrane. Mix and close tube.
2. Incubate in heating block at 80°C for 40 min.
3. Remove membrane from tube, and wash with 50 μL water.
4. Dry combined solutions in Speed Vac concentrator.
5. Resuspend in 25 μl and add 0.5 nmol lactobionic acid as internal standard for quantitation. Analyze by HPAEC-PAD using a linear gradient of 0–200 m*M* NaAcetate in 250 m*M* NaOH over 15 min (*see* **Notes 20–22**).
6. Compare retention time with standard of 1 nmol *N*-acetyl and *N*-glycolyl-neuraminic acids.

Table 1
Commonly Used Commercially Available Lectins

Taxonomic name	Common name	Specificity[c,d]	Source[a]	Form[b]
Aleuria aurantia	AAA	α-(1,6)Fuc	BM	F, D
Amanranthus caudatus	Amaranthin (ACA)	Galβ-(1,3)GalNAc	BM	F, D
Canavalia ensiformis	Jack bean Concancavalin A (Con A)	α-Man > α-Glc	BM, SCC, PH	F, D, B, H, A
Datura stramonium	Jimson week (DSA)	β-(1,4)GlcNAc terminal GlcNAc	BM, SCC	F, D
Maackia amurensis	MAA	α-(2,3)NeuAc	BM	F, D
Phaseolis vulgaris	Red kidney bean (PHA-L)	Complex N-linked	BM, SCC	F, D, B, A
Arachis hypogaea	Peanut (PNA)	Gal(1,3)GalNAc	BM, SCC	F, D, B, H, A
Ricinus communis	Castor bean (RCA120)	Terminal Gal	BM, SCC	F, D, B, H, A
Sambucus sieboldiana	Elderberry (SNA)	α-(2,6)NeuAc	BM, SCC	F, D
Triticum vulgaris	Wheat germ (WGA)	(GlcNAc)$_2$ > GlcNAc	BM, SCC, PH	F, D, B, H, A
Helix pomatia	Snail, edible (HPA)	Terminal GalNAc	SCC, PH	F, B, H, A
Lens culinaris	Lentil	α-Man, α-Glc	SCC, PH	F, B, A
Glycine max	Soybean (SBA)	GalNAc	SCC	F, B, P, A
Erythrina cristagalli	Coral tree (ECA)	Gal (1,4)GlcNAc	SCC	F, B, P

[a]BM, Boehringer Mannheim; SCC, Sigma Chemical Company; PH, Pharmacia.

[b]F, free (unlabeled); D, digoxigenin- (DIG) labeled; B, biotinylated; P, peroxidase-labeled; A, conjugated to agarose beads.

[c]GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Glc, glucose; Man, mannose; Fuc, fucose; NeuAc, *N*-acetyl neuraminic acid (sialic acid).

[d]D-sugars are the preferred sugars.

3.4.2. Analysis of Neutral Monosaccharides

1. Add 100 μL 2 *M* TFA to the desialylated membrane spots (*see* **Note 17**). Mix and close tube (*see* **Note 18**). Rewet membrane with methanol before addition of acid if dried out.
2. Incubate in a boiling water bath for 4 h.
3. Remove membrane from tube, and wash with 50 μL water. Keep membrane for subsequent amino acid analysis if required.
4. Dry combined solutions in Speed Vac concentrator.
5. Resuspend in 25 μL water, and add 0.25 nmol 2-deoxyglucose as internal standard for quantitation. Analyze by HPAEC-PAD eluted isocratically with 12 m*M* NaOH for 30 min (*see* **Notes 20–22**).
6. Compare with standard mixture containing fucose, 2-deoxyglucose, galactosamine, glucosamine, galactose, glucose, and mannose (5 μL of 1 m*M* solution) (*see* **Note 19**). This is the order of elution of the monosaccharides from the CarboPac column.

4. Notes
4.1. Periodic Acid/Schiff Staining

1. All steps in this procedure should be performed with shaking and should be carried out in a fume hood.
2. A negative result often means that insufficient protein is present or that the protein has little glycosylation.
3. Be aware that highly glycosylated proteins do not transfer well to nitrocellulose membranes and failure to detect may be due to inefficient transfer to the membrane. It is sometimes useful to carry out the stain on the gel after transfer to test for remaining glycoprotein.
4. This method can be used on PAGE gels, agarose, or polyacrylamide/agarose composite gels. The procedure should be carried out on an orbital shaker or rocker (an orbital shaker may cause background swirls on some gels). High background staining can occur when staining some batches of agarose or composite gels.
5. PAS staining after transfer of glycoproteins to membranes eliminates the need for extensive fixation steps, as well as shortening the time needed for washing steps, without loss of staining intensity. Results are easier to visualize and are easier to store. Before Western transfer, gentle rocking of the gel for 30–60 min in transfer buffer is suggested in order to remove SDS, which can lead to higher backgrounds during membrane staining.
6. PAS staining on nitrocellulose membranes increases sensitivity slightly and reduces washing times considerably compared with gel staining.

4.2. DIG/Anti-DIG AP Labeling

7. All steps in the procedure except for color development should be done on a shaker or rocker. Swirling background staining may occur when using a orbital shaker.
8. Prelabeling gives a higher sensitivity than postlabeling, requires less DIG (1 μL/ labeling), and can be used with <0.25% SDS, NP-40, Triton X-100, but not with

octylglucoside. Prelabeling results in broader bands than postlabeling and may result in a change of p*I* of the proteins in the first dimension. Prelabeling is negatively influenced by mercaptoethanol, DTT, glycerol, and Tris.

9. The postlabeling procedure is used for proteins that have already been separated by gel electrophoresis and immobilized on blots. It is important to use PBS (buffer E) instead of TBS (buffer A) in the initial stages, since Tris inhibits the DIG-labeling process.

10. Nitrocellulose and PVDF membranes can be used. Nylon membranes result in high background.

11. Postlabeling results in sharper bands than prelabeling, but has a lower sensitivity and requires more DIG (5 μL/gel).

12. For mucins and other heavily glycosylated proteins, it may be necessary to fix the bands to the membrane before labeling by washing in 1% KOH for 5 min. If this procedure is done, increase the initial washes to 3 × 10 min in buffer B.

4.3. DIG-Labeled Lectin Staining

13. Suggested dilutions of the DIG-labeled lectins: SNA: 1/1000, MAA: 1/500, DSA: 1/1000, PNA: 1/100.

14. The divalent ions are necessary for optimal lectin reactivity.

15. The binding of some lectins will be increased by desialylation, whereas the binding of others will be decreased.

4.4. HPAEC Analysis of Monosaccharide Composition

16. Data on the composition of both the acidic sialic acids and the hexoses and amino sugars can be obtained sequentially from a single spot. After the 4 *M* TFA hydrolysis, amino acid analysis (6 *M* HCl, 1 h, 155°C) can be performed subsequently on the same spot for identification of the protein.

17. If more accurate quantitation of the amino sugars is required, stronger hydrolysis using 4 *M* HCl for 4 h at 100°C can be carried out instead of the milder 4 *M* TFA hydrolysis. Subsequent amino acid analysis cannot be performed in this case.

18. It is important for monosaccharide analysis that the tubes be kept clean in a cellulose-free environment (i.e., no paper, cotton wool, or dust!) because of the common glucose contamination from the hydrolysis of cellulose and other ubiquitous polysaccharides. For the same reason, a blank of all reagents should be run to establish background contamination levels.

19. Glucose will almost always be present as a contaminant, so it is exceptionally difficult to quantitate if it is a constituent. Examples of the monosaccharide composition of typical N-linked sugars (fetuin) and O-linked sugars (mucin) are shown in **Fig. 1**.

20. The HPLC system must be totally metal-free because of the caustic reagents. All solvents must be made free of carbon dioxide by boiling of the Milli-Q water used. The water is cooled under argon, and the solutions kept under helium to reduce the precipitation of sodium carbonate from the sodium hydroxide solutions and the resultant effect on the anion-exchange chromatography.

21. The new DIONEX CarboPac PA10 gives better separation of the monosaccharides than the CarboPac PA1, but the latter may still be used
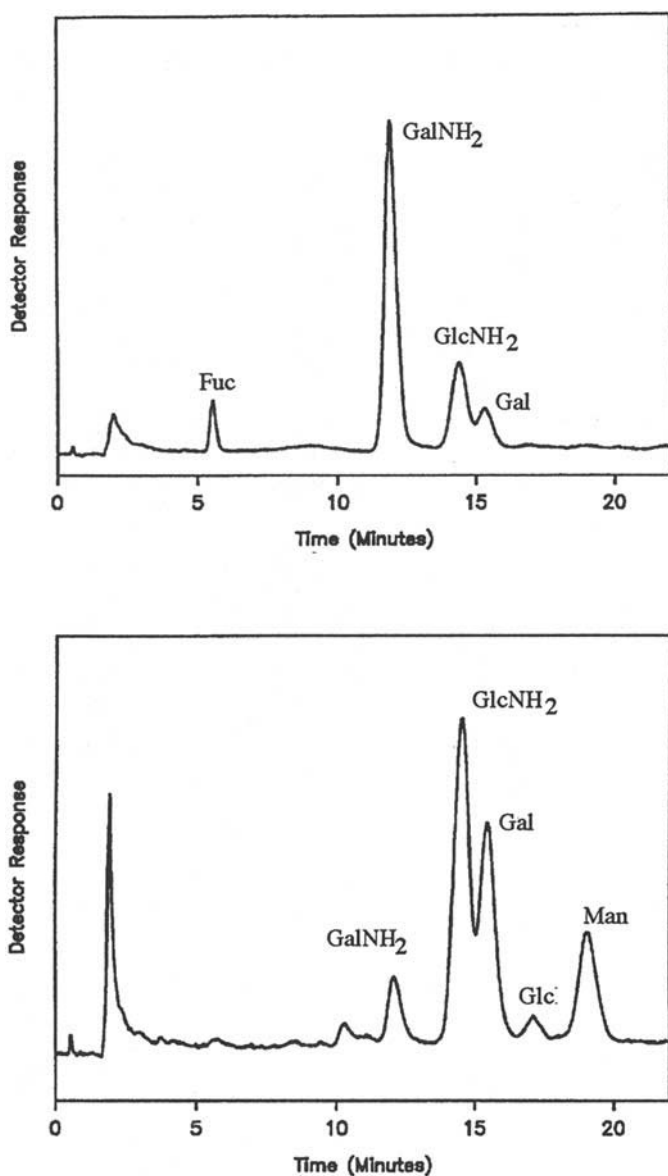
Fig. 1. Monosaccharide composition of glycoprotein hydrolysates. Upper, bovine submaxillary mucin; lower, fetuin. Separation on HPAEC-PAD.

effectively and can be used for both monosaccharide and oligosaccharide analysis.

22. The columns must be washed with high alkali (0.4 *M* NaOH) for 10 min and re-equilibrated between injections.

Acknowledgments

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39

Image Acquisition in 2-D Electrophoresis

Wayne F. Patton, Mark J. Lim, and David Shepro

1. Introduction

Prevalent methods for visualizing proteins resolved by two-dimensional (2-D) gel electrophoresis include autoradiography, silver staining, and Coomassie brilliant blue staining *(1,2)*. The organic dye Coomassie brilliant blue is capable of detecting as little as 100 ng of protein, but this is considerably less sensitive than silver staining or autoradiography *(1,2)*. Silver staining allows detection of low-nanogram amounts of protein, but detection sensitivity of autoradiography can be considerably better, since it depends principally on the specific activity of the radiolabeling method. Other staining techniques are valuable for specific applications in 2-D gel electrophoresis, such as Edman-based protein sequencing, mass spectrometry, and immunoblotting. Many of these applications require stains that do not covalently modify proteins, are relatively sensitive, and are easily reversible *(3)*. Additionally, immunoblotting protocols are often based upon the enzymatic formation of a colored formazan product or the chemiluminescent production of light *(4)*. Thus, image acquisition requires detection of diverse chromogenic, radioactive, and luminescent signals.

Two of the most common image-acquisition devices utilized in electrophoresis are charge-coupled device (CCD) cameras and document scanners. Phosphor storage imaging and multichannel array detectors are of more limited use, since they are employed almost exclusively to detect radioactively labeled proteins in 2-D gels *(5,6)*. A comprehensive review of image-acquisition devices should be consulted for complete descriptions of available hardware and technologies *(6)*. The CCD camera is perhaps the most versatile image-acquisition device for electrophoretic applications (**Fig. 1**). CCD cameras can be employed to acquire images of a variety of stained gels (silver, Coomassie brilliant blue,
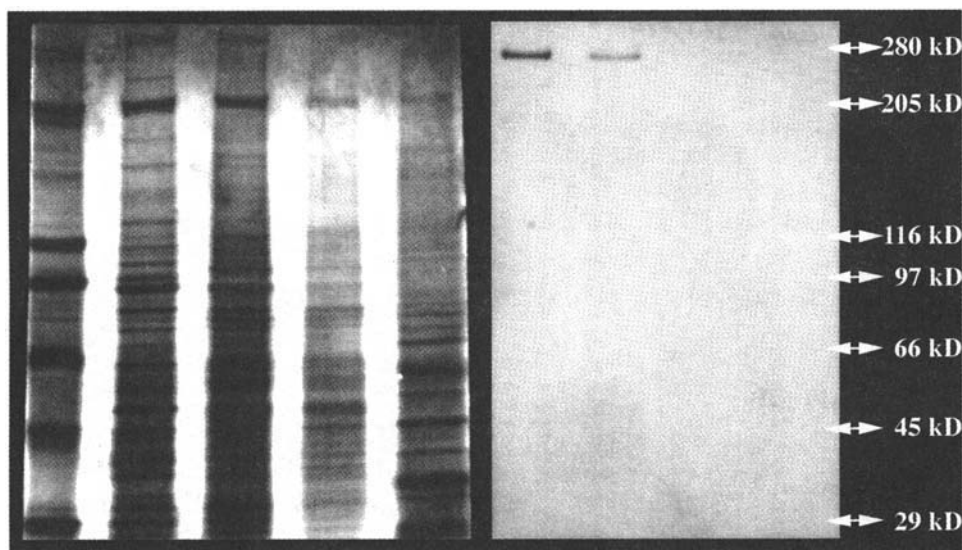
Fig. 1. Transmissive imaging of human omentum microvascular endothelial cell sub-
cellular fractions electroblotted to nitrocellulose membrane (**ref. *10***; *see* Chapter 8 for
fractionation procedure). Left image: Ferrozine/ferrous followed by ferrocyanide/ferric
double-metal chelate stain of total protein (*see* Chapter 37 for staining procedure). Lane 1:
High-mol-wt standards supplemented with chicken gizzard filamin. Lane 2: Cytosol frac-
tion. Lane 3: Membrane fraction. Lane 4: Nucleur fraction. Lane 5: Cytoskeleton frac-
tion. Right image: Same blot destained and reprobed with antifilamin antibody, followed
by alkaline phosphatase-conjugated secondary antibody. BCIP/NBT was used to visual-
ize alkaline phosphatase activity colorimetrically. Molecular-weight standard lane is
omitted in the second image. The electroblot is masked with black cardboard and imaged
according to procedures described in **Subheading 3.2.**

bathophenanthroline disulfonate-europium), autoradiographs, and stained
electroblots (metal chelate stains, BCIP/NBT immunoblots, colloidal gold
stain) *(5)*. The CCD camera depends on the same optical design as conven-
tional cameras, except that there is a CCD chip instead of photographic film in
the focal plane of the lens *(7)*. The CCD chip is a transistorized silicon crystal
light sensor on an integrated circuit. The silicon crystal is an array of atoms
whose bonds are broken by absorbing light of certain wavelengths. Breaking
these bonds causes release of electrons, but they are maintained in the local-
ized region of the chip until collected and read by the computer. The electri-
cally charged regions are interpreted by an analog-to-digital (A/D) converter
and translated into digital information. On digitization of a polyacrylamide gel
by a CCD camera, the image is stored onto the hard disk of a computer, image
intensities are discretely sampled, and the sampled values are quantized to a

discrete set of integer values. The CCD camera measures the intensity of light transmitted through the gel and records the logarithm of each value. The image is stored as a file consisting of rows of numbers (pixels). Each row of numbers consists of a string of numbers. For an eight-bit image, the numbers range in value from 0 to 255. The values for each number in the matrix represent the gray scale values of the digitized image.

The document scanner is a popular, low-cost device gaining widespread acceptance for image acquisition in electrophoresis. Document scanners consist of a 1-D array of detectors *(7)*. The array is essentially a "narrow CCD chip" that is physically moved across the gel. As the gel is scanned, light is reflected off the image and back to the CCD chip, which records the light intensity as described for the CCD camera. The computer gathers this continuous scan into a 2-D array of numbers. Generally, document scanners have more detectors along their length than the width of the standard 2-D array in the CCD chip. Changing magnification can be performed within the software package and involves changing the mechanical scanner speed. With the 2-D CCD camera, changing magnification involves physically changing the lens of the camera or adding extension tubes.

Phosphor storage imaging plates consist of fine phosphor crystals of BaFBr:Eu^{2+} in an organic binder *(6)*. High-energy radiation from the radiolabeled protein excites electrons of the Eu^{2+} ion into the conduction band of the phosphor crystals. The electrons are then trapped in F-centers of the $BaFBr^-$ complex with a resultant oxidation of Eu^{2+} to Eu^{3+} that constitutes the latent image of the radioactive sample. Exposing the latent image to 633 nm red light from a helium-neon laser releases the electrons from the F-centers in the lattice back to the conduction band of the crystal, reducing Eu^{2+} to Eu^{2*}. Eu^{2*} then releases a photon at 390 nm as it returns to the ground state. The intensity of the europium luminescence at 390 nm is measured and stored digitally in relation to the position of a scanning laser beam, resulting in a profile of the latent image formed on the storage phosphor plate by the original radioactivity from the sample. The residual image on the phosphor plate is erased by irradiation with visible light, and the plate is ready for a new exposure. The principal advantages of storage phosphor imaging are increased sensitivity, good spatial resolution of signal, and greater linear dynamic range of response.

The microchannel array detector directly detects radioactively labeled proteins in real time *(5,6)*. The microchannel array plate is a laminated structure with alternating conductive and nonconductive layers. A voltage step gradient is imposed on the successive conductive layers to produce an electric field of approx 600 V/mm. β-particles emitted by the sample pass through protective windows of the microchannels and ionize argon gas containing trace amounts of CO_2 and isobutane. The resulting electrons produced are accelerated by the

high electric field in the microchannel, causing further ionization of the gas. The electron cloud migrates up the electric field gradient into a multiwire chamber. The multiwire chamber consists of a plane of anode wires and two perpendicular planes of metallic cathode tracks, corresponding to *X* and *Y* coordinates. A further cascade amplification occurs in the high electric field around the anode wires, resulting in electric pulses in the *X*- and *Y*-cathode tracks. The resulting signals in the cathode tracks are digitized and transferred to a digital signal processor.

When selecting a 2-D image analysis system, the image-acquisition device should be a secondary consideration, as most analysis systems can be interfaced with most input devices *(8,9)*. Image acquisition is not image analysis, since the input device is simply taking a picture of the gel. Analysis is performed after image acquisition using image analysis software. Selection of an appropriate image-acquisition device depends on the types of detection systems routinely utilized to visualize proteins in the laboratory. The CCD camera is an excellent general-purpose acquisition device, since it permits analysis of a wide range of gels, membranes, and films. We find document scanners suitable for analysis of membranes, but more difficult to use with gels and autoradiographs. CCD cameras and document scanners are more susceptible to gray scale saturation effects than other detectors, such as laser-based scanners and multichannel array detectors *(5,6)*. The former are generally eight-bit digitizers with maximum usable optical density (OD) ranges of no more than 300 to 1. The response range of storage phosphor and multichannel array detectors are approx 10^5 to 1. Phosphor imagers and multichannel arrays are most commonly used with radioactive samples, though phosphor imagers may also be used for detection of some chemiluminescent signals. Phosphor imagers provide better spatial resolution than multichannel array detectors, but are also susceptible to signal fading.

Protocols for acquiring images of Coomassie brilliant blue, silver, autoradiographs, colloidal gold, BCIP/NBT-, Ferrozine/ferrous-, and bathophenanthroline disulfonate-europium-stained 2-D patterns using a CCD camera system are provided in the following sections. All protocols were developed on the BioImage computerized imaging workstation (Bio Image Products, Ann Arbor, MI), though attempts have been made to provide generic procedures.

2. Materials

2.1. Coomassie Brilliant Blue and Silver-Stained Gels.

Filters, calibration wedges, light source, and computer are from BioImage Products, Ann Arbor, MI.

1. Neutral density filters: 0.1, 0.3, 0.9 OD.
2. 600 ± 70 nm band-pass filter (Coomassie brilliant blue-stained gels).

3. 450 ± 70 nm band-pass filter (silver-stained gels).
4. Transmissive gray scale calibration wedge (11- or 21-step).
5. 45-W White light box.
6. Images of electrophoresis gels are obtained by digitizing gels at 1024 × 1024 pixels (picture elements) resolution with 256 gray scale levels using an eight-bit, Videx Megaplex black and white CCD camera equipped with Tokina 17-mm lens.
7. Computerized gel image analysis workstation.

2.2. Ferrozinel/Ferrous-, Ferrocyanide/Ferric-, and NBT/BCIP-Stained Blots

1. 600 ± 70 nm band-pass filter (optional).
2. 450 ± 70 nm band-pass filter (optional).
3. Transmissive gray scale calibration wedge (11-step or 21-step).
4. 45-W White light box.
5. Images of electroblots are obtained by digitizing gels at 1024 × 1024 pixels (picture elements) resolution with 256 gray scale levels using an eight-bit, Videx Megaplex black- and white-CCD camera equipped with Tokina 17-mm lens.
6. Computerized gel image analysis workstation.

2.3. Bathophenanthroline/Europium Stained Gels or Blots

1. 600 ± 70 nm band-pass filter.
2. Reflective gray scale calibration wedge (11-step or 21-step).
3. High-intensity UV light box (UVP, Upland, CA).
4. Images of gels and blots are obtained by digitizing gels at 1024 × 1024 pixel (picture elements) resolution with 256 gray-scale levels using an eight-bit, Videx Megaplex black and white CCD camera equipped with Tokina 17-mm lens
5. Computerized gel image analysis workstation.

3. Methods

3.1. Coomassie Brilliant Blue-Stained Gels, Silver-Stained Gels and Autoradiographs

1. Turn on the white light box, and allow it to warm up for 30 min.
2. For Coomassie brilliant blue-stained gels, attach a 0.3 OD neutral density filter and a 600 ± 70 nm band-pass filter to the lens. For silver-stained gels, place only a 450 ± 70 nm band-pass filter on the lens at this stage, whereas autoradiographs only require a neutral density filter (*see* **Notes 1** and **2**).
3. Place the sample on the light box, and adjust the camera height such that the sample maximally fills the field of view. Mask the edges of the sample with black poster board to block out extraneous light. Coarsely adjust the focus.
4. Adjust the exposure time and/or lens aperture (F-stop) to obtain the proper light levels. Most software packages include a light meter or brightness gage to aid in this step. If the light levels are too bright or dim, the full dynamic range of the CCD camera will not be fully utilized.

5. Remove the sample and place a transmissive gray scale step tablet on the light box. Mask the edges of the step tablet if it does not cover the entire light box. Adjust the focus on the lens to make the image as sharp as possible.

6. The BioImage system can determine pixel size from the step tablet for the purpose of calculating integrated intensities. Select the "size" option if determination of integrated intensities is required (*see* **Note 3**).

7. Remove the step tablet from the light box, and select the "equalize" option to correct for variations in the light source (*see* **Notes 4** and **5**).

8. Place the sample on the light box, and mask it to block extraneous light. Select the "scan" option to acquire the image (*see* **Note 6**).

9. The user should be prompted to place the gray scale step tablet on the light box (masked) and scan it to calibrate the sample image.

3.2. Ferrozine/Ferrous-, Ferrocyanide/Ferric-, and NBT/BCIP-Stained Blots

The following procedure is suitable for scanning colloidal gold-, Ponceau red-, BCIP/NBT-, amido black-, and Pyrogallol red/molybdenum-stained blots. Using this technique, opaque nitrocellulose membranes are scanned transmissively rather than reflectively. This approach offers certain advantages over conventional reflective imaging: With the metal chelate stains in particular, transmissive scanning significantly improves signal detection while maintaining a white background. Furthermore, in some cases, the protein sample penetrates through the membrane support to the reverse side (particularly with vacuum slot blotters). The metal chelate complexes stain proteins present throughout the thickness of the membrane and on both surfaces, but reflective scanning only detects signal on the front surface. This problem is alleviated with transmissive scanning. Depending on the application, however, reflective imaging may suffice.

1. Turn on the white light box and allow it to warm up for 30 min. Turn off any extraneous overhead lights for the remainder of this procedure.

2. Place the sample on the light box, and adjust the camera height such that the sample maximally fills the field of view. Mask the edges of the sample with black poster board to block out extraneous light. Coarsely adjust the focus of the CCD camera (*see* **Notes 7** and **8**).

3. Adjust the exposure time and/or lens aperture to obtain the correct light levels (*see* **Note 9**). Most software packages will include a light meter or brightness gage to aid in this step. If the levels are too bright or too dim, the full dynamic range of the CCD camera will not be fully utilized.

4. Remove the sample, and place a transmissive gray scale step tablet on the light box. Mask the edges of the step tablet if it does not cover the entire light box. Adjust the focus on the lens to make the image as sharp as possible.

5. The BioImage system can determine pixel size from the step tablet for the purpose of calculating integrated intensities. Select the "size" option if determination of integrated intensities is required (*see* **Note 10**).

6. Clear the light box, and select the "equalize" option to correct for variations in the light source (*see* **Note 11**).
7. Place the sample on the light box and mask it to block extraneous light. Select the "scan" option to acquire the image.
8. The user should be prompted to place the gray scale step tablet on the light box (masked) and scan it to calibrate the sample image.

3.3. Bathophenanthroline/Europium-Stained Gels or Blots, Scanned at 302 nm, UV-B

This procedure is also suitable for scanning ethidium bromide-stained gels. Low-light scanning software is required to allow for extended exposure times. Additionally, a reflective white light source is required for scanning both gels and blots. For blots, membranes are left opaque during the scanning procedure. Although techniques do exist to render opaque nitrocellulose membranes transparent, most are not compatible with the bathophenanthroline/europium stain. As a result, a UV lighting system capable of reflectively illuminating from above is required.

1. Attach the 600 ± 70-nm band-pass filter to the camera lens, and set the F-stop on the camera lens to a low value (approx 5.6).
2. Turn on the reflective white lights. Gels are placed directly on the UV light box. Blots are placed below the camera on a suitable flat surface (black).
3. Adjust the camera height such that the sample maximally fills the camera's field of view (*see* **Note 12**). Coarsely adjust the focus.
4. Remove the sample, and replace it with the reflective calibration step tablet.
5. Adjust the focus on the lens to make the image as sharp as possible.
6. The BioImage system can determine pixel size from the step tablet for the purpose of calculating integrated intensities. Select the "size" option if determination of integrated intensities is required (*see* **Note 13**).
7. Remove the step tablet.
8. Turn off reflective (white) and overhead lights. The room should be entirely dark for the UV scanning portion of this procedure. Proper protection should be worn to protect eyes and face from UV irradiation (polycarbonate safety faceshield, Fisher Scientific, Pittsburgh, PA).
9. Turn on the UV light source. Blots must be illuminated from the top with UV light (*see* **Note 14**). Membranes are illuminated using a high-intensity UV-B light source with maximal wavelength output of 302 ± 16 nm. Luminescence on excitation at 302 nm is three times more intense than at 254 nm and 12 times more intense than at 365 nm (UV-C or UV-A) *(11)*.
10. The light source may be equalized at this point (*see* **Notes 15**). This can be achieved by covering the light box with a uniformly fluorescent material (e.g., a sheet of overhead transparency film) and selecting the "equalize" option.
11. Remove any materials placed on the light table in **step 10**.
12. Place the sample on the light table, and position it correctly (use reflective white lights if necessary).

13. Turn off any reflective white lighting that was used in **step 12**.
14. Determine the correct exposure time using the prescan option. Some software packages may provide utilities for determining if any pixels in the image are saturated. If saturation is reached, reduce exposure time, and repeat the prescan.
15. Select the scan option to acquire the image.

4. Notes

1. For Coomassie brilliant blue-stained gels, the neutral density filter serves to block out excessive transmitted light, whereas the 600 ± 70 band-pass filter enhances the signal-to-noise ratio.
2. Some silver stain protocols produce spots that stain various shades of red. Spots that stain a very clear red rather than brown appear white when converted to a gray scale image. The 450 ± 70-nm band-pass filter eliminates this problem.
3. If the camera height or image dimensions are changed while scanning multiple samples, size data will have to be recollected to allow crosscomparison of gels in a database. If these parameters are not changed, size data will only have to be obtained once when scanning multiple samples.
4. Silver stain protocols often produce relatively dark backgrounds. To correct for this background, a combination of neutral density filters can be used at this step. The filters should approximate the background of the sample. After equalizing in the presence of the neutral density filters, the filters should be removed before continuing to the next steps.
5. When scanning multiple samples for crosscomparison in gel databases, the light source must be equalized every time the exposure time and/or aperture setting is changed.
6. Coomassie brilliant blue-stained gels may not quantify properly, even though the image may appear correct by eye. Coomassie brilliant blue gels often have an extremely clear background that allows excessive transmitted light to reach the camera. The resultant background may actually be a brighter white level than the zero OD reference on the step tablet. If the background levels fall below the absolute baseline (zero OD), the image will not quantify correctly. The use of a 0.1 OD Wratten neutral density filter during the scanning step may alleviate this problem. If the filter is used, it should be used for all samples whose images will be crosscompared in a gel database.
7. For reddish or purple stains, the use of a 450 ± 70-nm band-pass filter may enhance image contrast. Such stains include Ferrozine/Ferrous, colloidal gold, Ponceau red, Pyrogallol red/molybdenum, and BCIP/NBT. For blue stains, such as the Ferrozine/Ferrous, followed by the ferrocyanide/ferric stain, a 600 ± 70-nm band-pass filter may enhance the image contrast. However, filters appear to have less impact when imaging transmissively vs reflectively.
8. Because the membrane support blocks a substantial amount of transmitted light, care must be taken to mask the sample completely. Any transmitted light that passes around the edges of the sample can significantly skew the light meter or brightness gage readings.

9. Owing to the lower amounts of light passing through the membrane support, low-light scanning software may be required to allow for longer exposure times. Additionally, small F-stop settings may also be required to allow sufficient light to reach the camera.

10. If the camera height or image dimensions are changed while scanning multiple samples, size data will have to be recollected to allow crosscomparison. If these parameters are not changed, size data will only have to be obtained once when scanning multiple samples.

11. When scanning multiple samples for crosscomparison in gel databases, the light source must be equalized every time the exposure time and/or aperture setting is changed.

12. Temporarily placing a white sheet of paper on the UV light box may help visualize the gel under reflective white lighting. The gel can be separated from the paper by a piece of clear plastic to avoid adhesion.

13. If the camera height or image dimensions are changed while scanning multiple samples, size data will have to be recollected to allow crosscomparison in gel databases. If these parameters are not changed, size data will only have to be obtained once when scanning multiple samples.

14. A variety of setups may be possible for top illumination. One way is to simply tip the UV light box onto its side and place it as close to the sample as possible (without blocking the camera). Although this offers a simple way to convert the UV light box to top lighting, the UV box must be sufficiently strong, since the sample receives less direct illumination.

15. Equalization is not required, but is highly recommended for blots, since uniform top illumination is difficult to achieve.

Acknowledgments

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40

Computer Analysis of 2-D Images

Ron D. Appel and Denis F. Hochstrasser

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) images, when used to analyze protein expression in large sets of biological samples, are best processed by computer. In this manner, one can detect and quantify even the faintest spots, quantitatively compare 2-D images among each other, or identify protein expression changes across various sets of 2-D images, such as disease vs control, or different stages in a toxicological study *(1)*.

This chapter presents the operations that have to be performed using a 2-D image analysis software package, in order to detect and quantify protein spots on one or more 2-D images, to compare the protein expression in several such images, to create master 2-D gel images that contain data obtained with the above-mentioned software procedures or with other 2-D PAGE related techniques, such as identified protein names or pI and M_r values. Several commercial and public domain software packages exist to analyze 2-D images. Although these packages differ in various aspects, such as the computer platform they can be run on, the graphical user interface, or the file format, most of them have the functions necessary to perform the procedures described in this chapter. The detailed procedure described in **Subheading 3.** will thus be based on one such software package that is available for Unix, Power Macintosh, Windows 95, and Windows NT computers.

2. Materials
2.1. Hardware

1. Computer (*see* **Notes 1** and **2**).
   a. Sun Microsystems® SPARCStation 4 or better, 64 MB of memory, 1 GB of hard disk, and the Solaris 2.4 (or later) operating system (*see* **Note 3**).

b. Apple Power Macintosh®, 32 MB of memory and 1 GB of hard disk.
c. PC-compatible computer, 32 MB of memory and 1 GB of hard disk, MS Windows NT 4.0 (or later) or MS Windows 95 operating system.

2.2. Software

This consists of Melanie II 2-D PAGE software, Bio-Rad Laboratories, Hercules, CA *(2)* (*see* **Notes 4** and **5**).

3. Methods

3.1. Introductory Comments

The procedures described here all work with the Melanie II software, using images stored in the GIF or TIFF (8 or 16 bits) file formats, or images previously processed with Melanie II. This section explains how to analyze 2-D images using MelView, Melanie II's main program, focusing on the logic behind the sequence of operations *(2,3)*. Practical details, such as how to start the program or where to find specific options on the screen, can be found in the software's user manual.

This section describes the procedures using Melanie II for Apple Power Macintosh. Versions for the Sun and for Windows work in a similar way.

The MelView window is divided into three parts: the horizontal menu bar on the top, the vertical tool bar on the left side of the window, and the rectangular display area. You may use the tools to select objects in the display area, and then apply actions from the various menu options to the selected objects. Objects are gel images, landmarks associated to specific points on the image, features (detected spots), labels associated to features, feature pairs between two gels, or groups of features across several gels. Objects on screen may be displayed, manipulated, or processed using the various options from the MelView menus. Prior to choosing an option from the menu bar, individual objects have to be selected using the tools provided in the left-hand side tool bar (**Fig. 1**).

3.2. Image Manipulation and Visualization

This section describes how to load images into Melanie II and how to perform actions that help in visually analyzing the 2-D images.

1. Open images: Click on the File menu, and select the Open option. The Open window will be displayed (**Fig. 2**). Click on each of the desired gel names, and each time click on the Add button or just double click on each gel name. This adds the requested gels to the Open gel box. Click on the Done button. The gels will be opened.
2. Select gels: To perform the actions described in the remaining part of this chapter, you must first select one or more gels. To select one gel, pick the Select tool in the tool bar. Put the cursor in a gel, and click on the mouse button. To select
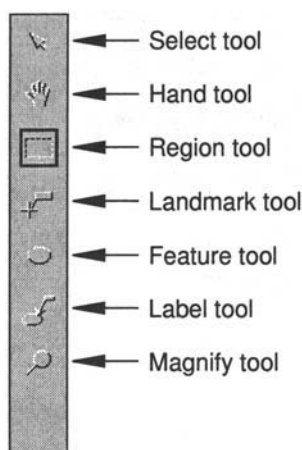
Fig. 1. The MelView tool bar.
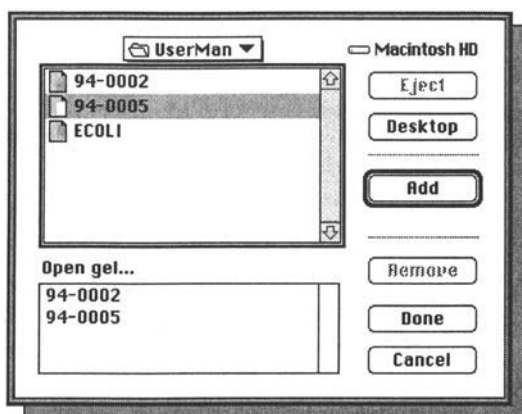
Fig. 2. The Open window allows the selection of the gels that will be opened and loaded into Melanie II.

more than one gel, select the first one as described above, then hold the shift key on the keyboard, and select additional gels by clicking on them. Alternatively, you may select gels using one of the options in the Select menu.

3. Show gels: Once you have opened a given number of gel images, you can decide which ones you want to display and which ones you prefer to hide at a given point in time. The Gels option in menu Show provides various choices to this purpose.

4. Zoom gels: You may zoom into an image to enlarge it or zoom out to see more of the gel image. Select the gels you want to zoom, then take *Select → Select Zoom*, and choose the zoom mode: 1 is the default mode and displays images in true size; 2 zooms in by a factor of 2; ½ and ¼ zoom out by a factor of 2 and 4, respectively.
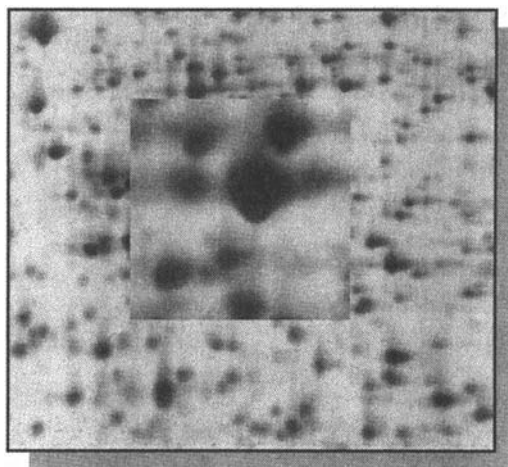
Fig. 3. The magnifying glass.

An alternative way to enlarge parts of gels is to use the magnifying glass. Pick the Magnify tool, and then hold down the mouse button. Similar to a magnifying glass, you may then move the mouse to change the zoomed region (**Fig. 3**).

5. Adjust gray levels: Modern scanners are usually able to scan 2-DE images with 12 or even 16 bits/pixel, that is, with 4096 or 65536 gray levels, respectively. Because common screens are only able to display 256 colors or gray levels, mapping has to be determined between the 4096 and 65536 gray levels, respectively, and the 256 screen gray levels. By default, MelView uses a linear mapping function, where the lightest point in the image is mapped to 0 (white) and the darkest point is mapped to 255 (black). You may choose other mapping functions, in order to highlight small light spots, for example. Select the Region tool, and draw a small rectangle in one of the selected gels by putting the cursor at the top left point of the region, holding down the mouse button and dragging the cursor to the bottom right point. Release the mouse button. The selected region will be delimited by a rectangle. In the Select menu, choose *Select Mode → Adjust Colors*. Each time you change one of the mapping parameters (the function or a slider), the change will immediately be reflected in the selected region (**Fig. 4**). To highlight small faint spots, choose a logarithmic mapping.

6. Stack gels: In given situations, it is helpful to stack two gels, that is, to display one gel on top of the other. By switching from one gel to the other, you may then better compare given gel areas. Select two gels, and then *Stack → Stack Selected Gels*. The two gels will be displayed on top of each other. To switch from one gel to the other, choose *Stack → Front To Back*, or just Command Key-F. By pressing Command Key-F quickly several times, the differences between the two gels can be observed visually. To unstack the gels, choose *Stack → Unstack Selected Gels*.
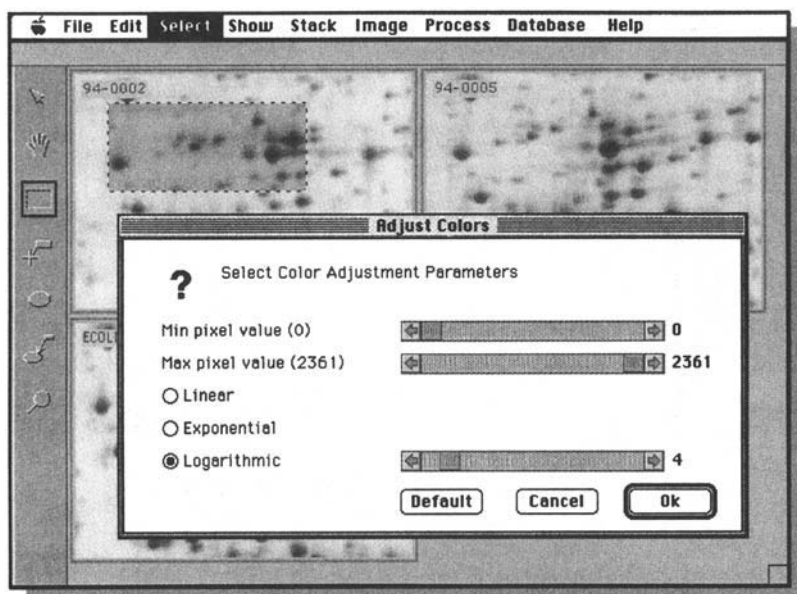
Fig. 4. The Adjust Colors option. The modifications are directly reflected in the selected region.

7. Save gels: Select all gels, and then choose *File → Save → Save Changes* to save all changes performed on the selected gels.

3.3. Spot Detection and Quantification

In Melanie II, the elementary component of a gel is the feature. Features are spot shapes automatically detected by Melanie II's feature detection algorithm. Each feature in a gel has an associated feature ID (a unique sequential number) automatically given when it is created. Features may be quantified, that is, their optical density (or instrument count for nonoptical images), area, and volume are computed. Alternatively, features may be modeled by Melanie's Gaussian modeling algorithm. In this case, their quantification values will be the bi-Gaussian peak values and volume, as well the area of the Gaussian's half-height section. Follow these steps:

1. Move the gels to an area of interest: Use the Hand tool to move the gels to an area of your choice.
2. Select a region: Select the Region tool, and draw a small rectangle in one of the selected gels, using the mouse and the button. In the Process menu, choose Detect Features. The Detect Features window pops up. Various detection parameters are available and may be adjusted. The default parameters have been optimized for silver-stained 2-D PAGE images (*see* **Note 6**).
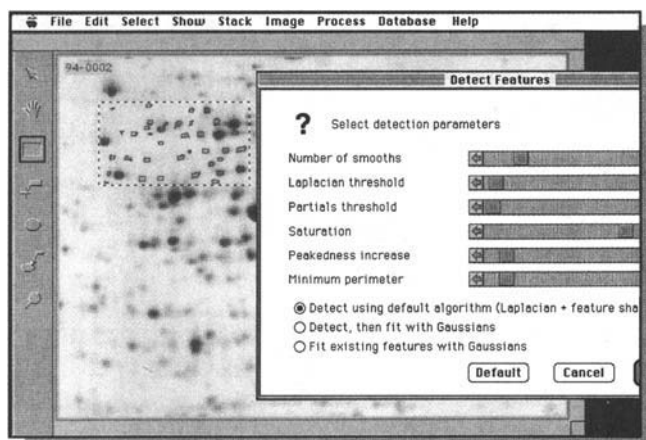
Fig. 5. Adjusting detection parameters.

3. Set parameters: By clicking Default, you can detect the features with the default parameter values in the small region, or you may adjust the following parameters. You can interactively see the changes made in the subregion as soon as you change any parameter (**Fig. 5**):
   a. Number of smooths will smooth the image before detecting features. Select the number of times to perform the smoothing.
   b. Laplacian threshold looks for peaks in the image. Set a small value to detect as many as possible.
   c. Partial threshold checks the spot curvature in both *x*- and *y*-axes to separate features.
   d. Saturation artificially makes peaks for saturated features. A value of 90% is usually considered correct.
   e. Peakedness increase is an interval value for how much Melanie II increases the curvature of saturated pixels.
   f. Minimum perimeter is the minimum feature size you decide to keep, ignoring smaller ones.
   g. Gaussians is opposite to the feature shape detection. This function will model the spots as bi-Gaussians and draw elliptic shapes.
4. Detect features. Click OK in the Detect Features window. The parameter values you have set will be used to detect the spots in all the selected gels (*see* **Note 7**).
5. Quantify features. Choose *Process → Quantify Features* to compute the feature values. Depending on the scanner that was used to digitize the images, the following quantification values are calculated for each detected feature (*see* **Notes 8** and **9**):
   a. OD—the highest optical density in the feature, or count—the highest instrument count in the feature for nonoptical images (autoradiography, chemiluminescence and so forth) (*see* **Note 10**).

Fig. 6. Report on features.

    b. AREA—the feature's area.

    c. VOL—the feature's volume, that is, the integration of OD (resp. Count) over the feature's area.

    d. %OD (resp. %Count)—the relative OD (resp. Count), that is, the OD (resp. Count) divided by the total OD (resp. Count) over the whole image.

    e. %VOL—the relative VOL is the VOL divided by the total VOL over the whole image.

3.3.1. Using Spot Values

Once spots have been detected and quantified, you can select all or some of them, and display a report on their computed values.

1. Select features: Features may be selected with the Features tool. Click on any feature to select it. The selected feature will be highlighted in green. To select more than one feature, select the first one by clicking on it with the mouse button, then hold down the shift key on the keyboard, and select additional features by clicking on them. To select all features in a given region, position the cursor at the top left position of the desired region, hold down the mouse button, and then drag the cursor to the bottom right position. All features in the selected region will be selected and highlighted in green.

2. Display report on selected spots. Choose Show → Features → Report On Features. A report will be displayed giving specific information on the selected features, such as features ID, quantification values, *pI*, and M_r, as well as the following data from the master gel, provided the selected gels have been matched to a master gel (*see* **Subheading 3.5.**): serial number, p*I*, M_r, and accession number (M:S/N, M:pI, M:Mw, and M:AC, respectively). The report may be saved or printed (**Fig. 6**).

3.4. Gel Matching

Gel matching is a key operation in 2-DE gel image analysis. The basic gel-matching algorithm consists of comparing two gel images and finding pairs of related features, that is, features depicting the same protein in both gels *(4)*. A

pair is represented by a doublet (f_1, f_2), where f_1 is a feature ID in the first gel and f_2 a feature ID in the other gel. One generally says that gels have been matched, and that individual features have been paired.

One can match two or more gels. Matching two gels means finding all the pairs between features of the two gels. Matching several gels means picking out a reference gel, and then successively matching each gel to the reference gel. In this way, features in all gels may be compared with features in the reference gel. For consistency, when matching only two gels, one usually also has to specify which one is the reference gel.

All features in selected gels that are paired with the same feature in the reference gel form so-called groups. The concept of group is essential to analyzing features in a set of gels. Elaborate reports, histograms, or data analysis may be produced from them.

Matching algorithms can produce either single pairs or multiple pairs. Matching with single pairs means that one feature in a gel will be paired to exactly one feature in the other gel. Matching with multiple pairs allows one feature in one gel to be paired to several features in the other gel.

Gel matching is performed in two main stages: gel alignment, where gel images are adjusted pixel-wise in order to be superimposable, and the matching process itself.

1. Create landmarks: The first step consists of verifying that all gels you are going to match contain at least four landmarks and that they represent the same points in all gel images. The best way to define these reference points is to set landmarks in the center of a few spots corresponding to known proteins, and to name the landmarks accordingly. Only landmarks with identical names in all selected gels will be used for the alignment. Decide which gel will serve as the reference gel. Choose four to seven points in this gel and set landmarks on it. Using the Landmark tool, double click in the center of the first spot you are going to put a landmark in. The landmark window will appear. Enter a name for this landmark (for example, L1), and click OK. The landmark will be shown on the gel. Repeat this procedure four to seven times in order to set several landmarks in your reference gel. Similarly set corresponding landmarks on the other gels. Make sure that corresponding landmarks are identified by the same name.

2. Align gels: Select all gels that have to be aligned. Choose *Process → Align Gels → Align Gels*. When requested, select the reference gel among the list of gels. Click OK. The selected gels will be aligned according to the reference gel, so that corresponding landmarks are best superimposed (*see* **Notes 11** and **12**).

3. Match landmarks: Select the gels, and then *Process → Match → Match Landmarks*. MelView will ask you to point to the reference gel. It will then, for each feature in the reference gel that has a landmark defined in it, pair it with features in the other gels that have the same landmark defined in them. These newly created pairs may then be used as starting pairs for matching (*see* **Notes 13** and **14**).
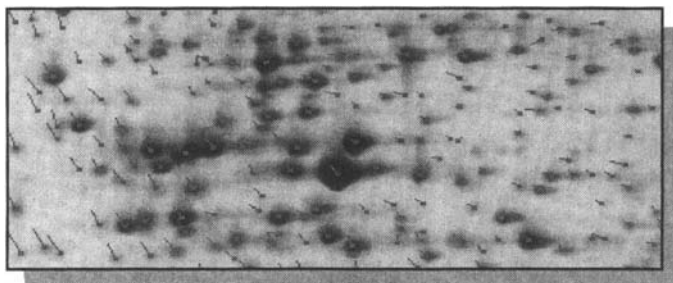
Fig. 7. Matched gels: Blue vectors indicate paired features.

4. Match gels: Choose *Process → Match → Match Gels*. MelView will first request you to designate the reference gel among the selected gels. Pick one of the gels, and then click OK. You will then be asked whether you allow multiple pairs. If you accept multiple pairs, press OK. If you want only single pairs, then click *No*. All gels will then be matched to the reference gel (*see* **Note 15**).
5. Save pairs: Choose *File → Save → Save Pairs*, or *File → Save → Save Changes* to save the result of the matching operation (*see* **Notes 16** and **17**).

3.4.1. Using Gel-Matching Results

There are mainly three ways of looking at the result of the matching process: in stack mode, highlighting all pairs, by selecting given spots and highlighting the corresponding ones in the other gel images, and by producing a report on paired spots.

1. Show pairs in stack mode. Select two gels that have been matched using the Select tool, and then *Stack → Stack Selected Gels*. The two gels will be displayed on top of each other. To switch from one gel to the other, choose *Stack → Front To Back*, or just Command Key-F. Choose *Show → Pairs → Show Pairs*. The pairs between the two stacked gels will be highlighted in the form of blue vectors linking the locations of paired features. The head of the vector points to the feature in the front gel (**Fig. 7**). To hide vectors, choose *Show → Pairs → Hide Pairs*.
2. Show selected pairs. To observe paired features when gels are in tile mode, you have to select the pairs. First select matched gels. Using the Feature tool, select a number of features in one of the gels. Choose *Select → Pairs → For Features*. Features in selected gels that have been paired to the selected features will be highlighted. If you have selected features in the reference gel, then all pairs for the given features will be selected in the other gels.
3. Report on pairs. You may display, print, or save a list of pairs between two gels. Select two matched gels and a number of pairs, using the Select tool. Take *Show → Pairs → Report On Pairs*. You will get the list of all paired spot IDs (identifications) between both gels. Another way to display the matching result is to select two or more matched gels, and then *Show → Gels → Report On*
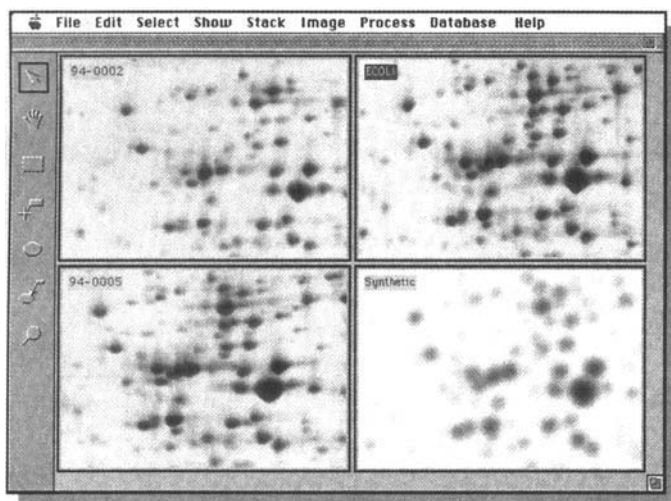
Fig. 8. A synthetic gel and its three contributing gels. The reference gel was ECOLI.

Matches. This will display the number of pairs between all the selected gels and any open gels.

3.5. Creating a Synthetic Gel

One of the best ways to study the variation in protein expression among a series of gels is to produce a synthetic gel by merging spots (features) from the studied gels, then to match all gels to the synthetic one, and finally to produce reports or histograms, or to analyze the matching results using data analysis or classification tools.

To create a synthetic gel, perform the following steps:

1. Select gels. Decide what gels will be used for creating the synthetic gel. Select them using the Select tool. The synthetic gel will contain features from these gels (*see* **Note 18**).
2. Select Reference gel. From these gels, pick one out as the reference gel. The synthetic gel will be based on it. This means that features will first be taken from the reference gel, and then missing features will be extracted from the other gels. The resulting synthetic gel will most likely look similar to the reference gel. You should therefore choose a good-quality gel image for the reference gel.
3. Match gels. Pair-wise match all gels chosen in **step 1**. For example, if you chose the three gels GelA, GelB, and GelC, and picked GelA as the reference gel, you will have to match GelA to GelB, GelA to GelC, and GelB to GelC.
4. Create synthetic gel. Choose *Image → Create Gels → Create Synthetic Gels*. When requested to do so, pick the reference gel and click OK. The Create Synthetic Gel window then lets you specify the new file name. Click OK.

The resulting synthetic gel will be created and displayed (**Fig. 8**).

3.5.1. Using a Synthetic Gel

The synthetic gel is useful as a reference gel to match a set of gels, because it contains the most important spots from all gels in the set. Once a synthetic gel has been obtained, match each of your gels to it. This will associate each matched spot to one spot in the reference (synthetic) gel and therefore produce a unique numbering scheme for the spots contained in all considered gels. The set of matched spots across all gels may then be considered as a matrix, where each column represents one gel and each line one spot labeled by its ID in the reference gel. You may then use one of the analysis or classification tools described in **Subheading 3.6**.

3.6. Classification Tools

Once you have matched a number of gels to a reference gel, you may analyze the change in protein expression over the set of gels using one of the following methods *(5)* (*see* **Note 19**).

3.6.1. Displaying Spot Groups

Melanie II defines a feature group as the set of features (spots) that represent the same protein across a given set of gels. You may select individual feature groups and display their respective quantification values, either numerically or graphically (*see* **Notes 20** and **21**). To select the groups that one or more features belong to, select features using the Feature tool, and then *Select → Groups → For Features*. The following two options are then available:

1. Reports on groups: Choose *Show → Groups → Report On Groups*. Melanie will ask you to pick out the reference gel. You then have to select one of the feature value types for the report (optical density [OD] or instrument counts [Count], spot volume [VOL], spot area [AREA], relative optical density [%OD], relative spot volume [%VOL], or relative instrument counts [%Count]). The report shows information on each selected group, such as group ID and value of each feature in the group. You may save or print the report.
2. Histograms on groups: A more visual way to look at groups is to display a histogram. To get histograms on groups, choose *Show → Groups → Histograms On Groups*. (**Fig. 9**).

3.6.2. Differential Analysis

This facility lets you automatically highlight spots that are differentially expressed between gels in two predefined classes. This option, as well as the next one (**Subheading 3.6.3.**), performs data analysis on spot groups contained in two predefined classes of gels. You must, therefore, prior to using any of these actions, define at least two classes of gels:
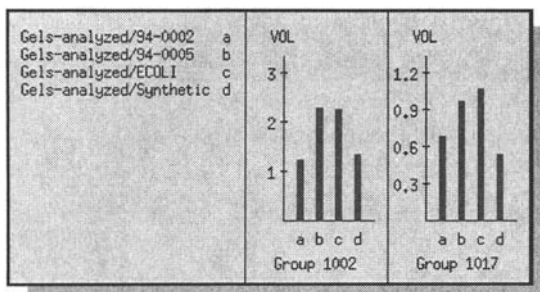
Fig. 9. Histograms for two spot groups.

1. Set first class. Select all gels that belong to the first class using the Select tool. Choose *Analyze → With Classes Set → Set Class*, and enter a name for the class. Click OK.
2. Set second class. Select all gels that belong to the second class using the Select tool. Choose *Analyze → With Classes Set → Set Class*, and enter a name for the class. Click OK.
3. Select spot groups. Using the Feature tool, select all spot groups on which the analysis will be performed. You may select features in one gel, and then *Select → Groups → For Features*. Alternatively, choose *Select → Features → All Features*.
4. Choose spot value. Choose *Analyze → With Classes Set → Differential Analysis*, and select the value type you want to perform the analysis on (OD, VOL, and so forth). Click OK.
5. Select ratio. In the pop-up window, move the slider so that the ratio of your choice is displayed. The slider range is 0–100%, with a default value of 50%. The default value means that all the selected groups in one class having a value greater than or less than at least 50% of the corresponding value in the other class will be selected. One hundred percent means that only the similar groups will be selected from the initial groups. Click OK.
6. Display results. Choose *Show → Analysis → Report on Analysis* to see the result of differential analysis. Additionally, all spots that differ between the two classes by the factor (ratio) you have chosen will be selected in the gel images, that is, they will be highlighted in green.

3.6.3. Student's t-Test

You may perform a Student's *t*-test on the spots contained in gels separated in two classes:

1. Set first class. Select all gels that belong to the first class using the Select tool. Choose *Analyze → With Classes Set → Set Class*, and enter a name for the class. Click OK.
2. Set second class. Select all gels that belong to the second class using the Select tool. Choose *Analyze → With Classes Set → Set Class*, and enter a name for the class. Click OK.

3. Select spot groups. Using the Feature tool, select all spot groups on which the analysis will be performed. You may select features in one gel, and then *Select → Groups → For Features*. Alternatively, choose *Select → Features → All Features*.
4. Choose spot value. Choose *Analyze → With Classes Set → Student's* t-*test*, and select the value type you want to perform the analysis on (OD, VOL, and so on). Click OK.
5. Select *t*-test value. In the pop-up window, move the slider so that the *t*-test value of your choice is displayed. The slider ranges from 0.0 to 1.0, with a default value of 0.95. Click OK.
6. Display results. Choose *Show → Analysis → Report on Analysis* to see the result. All spot groups with a *t*-test value greater or equal to the value set in **step 5** will be selected in the gel images, that is, they will be highlighted in green. The *t*-test value of each selected group is also displayed in a separate report window.

3.6.4. Correspondence Analysis

This lets you perform correspondence analysis on selected spot groups and all the visible gels, and try to group the gels into different classes *(6)*.

1. Select spot value. Choose *Analyze → Without Classes Set → Correspondence Analysis*. Select the value type you want to perform the analysis on (OD, VOL, and so forth). Click OK.
2. Select number of groups. In the pop-up window, move the slider so that the number of group values of your choice is displayed. This lets you decide the number of most significant spots that will be displayed in the resulting graphic. The default value is 5. Click OK.
3. Display results. Choose *Show → Analysis → Report on Analysis* to see the numerical results. Choose *Show → Analysis → Graphics on Analysis* to display a graphic with the projection of the gels and spots on the two most significant axes of the factorial space. The closer the gels are displayed on the graphic, the more similar they are. The most significant spots are displayed closer to the group of gels they characterize best.

3.6.5. Exporting the Data

The matrix formed by the data after matching a set of gels to a reference gel may alternatively be exported to an ASCII file in order to be processed using third-party data analysis tools. The ASCII file is a table with tabulator separated columns. Each column represents one gel. The lines contain spot data, one group per line:

1. Select spot groups. Select spot groups using the Feature tool or any option in the *Select → Features* menu.
2. Export data. Choose *File → Export*, and specify Excel Table.
3. Select the reference gel. Pick the reference gel for the groups.
4. Select spot value. Pick one value type (OD, Count, VOL, AREA, %OD, %VOL, %Count).

5. Select options. Decide if you want to include data from the reference gel or only from the other gels. If the reference gel is a synthetic one, it is recommended not to include related data into the exported file. Otherwise you may decide to include the reference gel's data into the exported table. Specify the file name. Click OK.

You may now import the data just produced into any third-party data analysis software.

3.7. Creating a Master Gel

Since powerful protein identification techniques are now available (*see* Chapter 42), it is important that 2-D maps can be annotated, especially to associate spots with their corresponding protein names. One straightforward way to accomplish this is by building a master gel. Such a master protein map possesses the same characteristics and properties as a normal 2-D gel image, and can therefore be processed in the same manner as described above. In addition, a label is associated with each spot for which the corresponding protein has been identified, which holds information specific to the protein, such as the protein name. Although any character string may be contained on a label, it is custom any to include the protein accession number (AC) taken from the SWISS-PROT protein sequence database *(7)* as well as its name in short format (*see* **Note 22**). This allows one not only to keep protein identification data directly linked to the protein map, but also to relate spots on the 2-D map to local or remote 2-D and protein databases, such as the SWISS-2DPAGE database *(9–12)* (*see* Chapter 41). Once you have created a master gel, you may also link the information it holds to any other 2-D gel by using it as the reference gel when matching gel images, and then automatically copying related data from the master to any other matched gel (*see* **Subheading 3.7.1.** and **ref. 8**) (*see* **Note 23**).

It is also useful to include pI and M_r data into the master, so that pI and M_r may be estimated on other gels that have been matched to the master. To establish a master gel, perform the following steps:

1. Create a label. To create a label, pick the Label tool, and double click on a feature you wish to associate a label to. The Label window pops up. Fill in the AC (accession number) and name fields. Click OK. The label is created and its AC is displayed on the gel. Repeat this step for all identified spots (*see* **Notes 21** and **22**).
2. Display protein names. Choose *Show → Labels → Show Name* in order to display protein names on the labels, instead of the AC (**Fig. 10**).
3. Add pI/M_r values. Select a point on the master image, for which you know the pI and M_r values, for example, the center point in an identified protein spot. Create a landmark as explained in **step 1** of **Subheading 3.4.**, and enter the pI and M_r values. Click OK. Repeat for a number of other points spread over the gel.
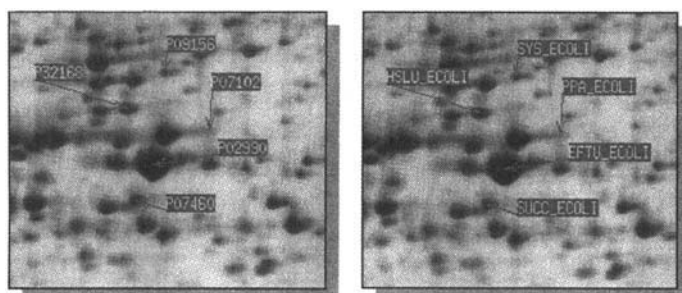
Fig. 10. Labels on a master gel—with ACs (left)—with names (right).
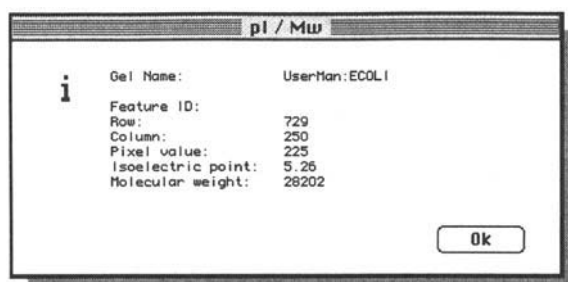
Fig. 11. The ID and pI/Mw window.

3.7.1. Using a Master Gel

A master protein map may be used in several manners. The simplest one is to use it as a stand-alone gel to retrieve the data it contains:

1. Open gel. Choose *File → Open*, and select a master gel.
2. Display labels. *Show → Labels → Show Labels* will display all the master's labels (*see* **Note 22**).
3. Display label names. Choose *Show → Labels → Show Name* in order to display protein names on the labels, instead of the AC.
4. Display pI/M_r information. If you have defined a few landmarks in the master and given pI and M_r values, you can display the interpolated pI and M_r values at any point in the gel. Take *Show → ID & pI/Mw*. This will display a small window holding the pI and M_r at cursor position. Moving the cursor will change the window content accordingly (**Fig. 11**).

One efficient way to use the master gel is to copy its data into other gels, in order to identify protein spots by gel comparison.

1. Match gels to master. To copy the master's information to some given gels, first match these to the master, following the procedure described in **Subheading 3.4.**

2. Copy data to gels. Select one or more features (spots) in any gels, and then take *Database → Labels → Add From Master*. Melanie II will look for features in the master that are paired to the selected ones. It will then copy their associated labels to the selected features. The selected features will thereafter have the same labels as their paired features in the master.

You may now use the gels as if they were masters (*see* **Notes 22** and **23**).

You may also link any identified spot on the master gel to a remote 2-D or protein database (*see* Chapter 41) (*see* **Note 23**).

4. Notes

1. The computer equipment described in **Subheading 2.1.** represents the minimal requirements necessary to perform computer analysis of 2-D images as described in this chapter. Any more powerful computer that meets these requirements can of course also be used satisfactorily.

2. The memory requirements given in **Subheading 2.1.** are to be regarded as the minimum memory necessary to run the software. In order to perform 2-D analysis efficiently, one should count at least 5 MB of memory or swap space (the virtual memory space available on a computer)/2-D gel (1000 × 1000 pixels and 12 or 16 bits/pixel) that is to be open at one time. For example, if you plan to analyze a set of 12 such images simultaneously, you should equip your computer with at least $12 \times 5 = 60$ MB of memory or swap space. We recommend to start using the software with at least 64 MB of memory and eventually adding supplementary memory at a later stage if needed. On Power Macintosh computers, we recommend not to use virtual memory.

3. On Sun Microsystems equipment, the Melanie II 2-D PAGE software can also be run under the SunOs 4.1.x or later operating system, but new Sun computers can currently only be purchased preloaded with the Solaris operating system.

4. The disk space necessary to run Melanie II 2-D PAGE is minimal (about 20 MB). The total disk space required is a function of the number of 2-D images one plans to store on disk. For 1000 × 1000 pixels and 12 or 16 bits/pixel images, count 2.5 MB/image. For example, a 2 Gigabytes hard disk on which 500 MB are used by the system can store 600 such images.

5. The Melanie II 2-D PAGE software is available for PC (Windows 95 or NT), Sun (Solaris), and Power Macintosh. The version you purchase must of course match your computer and operating system. Details about the software may be obtained at the following URL: http://www.expasy.ch/melanie/melanie-top.html

6. Detecting features (spots shapes) is a key and often difficult step, because it is subject to changes in the image characteristics that depend on the gel image itself. To obtain the best spot detection, use only images that show a high dynamic range, that is, 12- or 16-bit images (4096 and 65536 gray levels, respectively). To determine the optimal detection parameters, detect spots in a small region defined with the Region tool, and first adjust the Laplacian threshold.

7. The default spot detection parameters have been optimized for silver-stained gels. Other staining techniques will request that you modify the parameters. In images

with a high dynamic range spots will be detected more easily. If spots are very faint, you might have to extend the dynamic range of the image using the *Image → Filter* option.

8. Quantification values reflect protein concentration only to some extend. They have been generated from image data and may vary according to a number of factors, such as type of scanner, scanning precision, and so forth. They should therefore be interpreted with care.

9. In images stained with methods, such as silver staining, different proteins may have different responses to the stain, and spot intensity function may vary from protein to protein. Quantification values can thus only be used to compare corresponding spots across gels.

10. Although the best quantification value (OD, VOL, and so on) can vary according to the selected spot, the overall most reproducible value is the optical density (OD), especially in silver-stained gels.

11. Gel alignment, where, prior to matching, gel images are adjusted pixel-wise in order to be superimposable greatly facilitates and speeds up the matching process. Nevertheless, it is optional. When very similar gels are being matched, this step may be skipped, but if matching fails or does not produce the expected results, one may still have to perform gel alignment.

12. Gel alignment may be used to superimpose and match gels obtained with different experimental conditions, such as narrow- vs wide-pH range IPG gels.

13. Gel-matching results depend on the quality of the gel images that are being matched, and also the degree of similarity between the gels. Very similar gels may be matched without setting any landmarks. The more dissimilar the gels, the more landmarks are needed to start the matching process. It is good practice to define four to seven landmarks before matching, but if the gels only weakly look alike, then several tens of landmarks may be necessary.

14. If you have set corresponding landmarks in all gels you want to match, you may pair the spots associated to these landmarks using the *Process → Match → Match Landmarks* option. Similarly, if you have assigned labels to given spots (for example, to spots corresponding to identified proteins), you can pair these spots using *Process → Match → Match Labels*. These pairs may then be used as starting pairs to improve and speed up the matching process greatly.

15. During gel matching, you must decide if you will allow multiple pairs, that is, to allow one feature in one gel to be paired to several features in the other gel. The advantage of allowing multiple pairs is that it lets the matching program associate one possibly poorly separated spot in one gel to two or more well-separated spots in another gel. You have, nevertheless, to be aware that multiple pairs increase the likelihood of obtaining erroneous matched pairs.

16. Saving pairs creates a pairs file that resides in the same folder as the matched gels. Therefore, only gels that reside in the same folder may be matched against each other.

17. In addition to selecting pairs corresponding to given features, several other possibilities exist to look at pairs, including the options to select only multiple pairs or only potentially badly paired spots. Refer to the user's manual for further details.

18. When selecting gels from which to create a synthetic gel, you should note the following points: the synthetic gel will likely resemble the reference gel. Therefore, care must be taken when choosing the reference gel. In order to include into the synthetic gel spots from as many gels as possible when analyzing gels from several populations or classes of gels, it is good practice to select between one and three representative gels from each population or class, depending on the number of classes. The total number of gels used to create the synthetic gel should lie between 3 and 7. The more gels are taken into consideration, the more complete the resulting synthetic gel will be, but be aware that all these gels must be matched pair-wise. Pair-wise matching of a large number of images may take time. For example, pair-wise matching three gels means that a total of three matches have to be performed. To pair-wise match 7 gels, 21 matches have to be carried out, and with 12 gels, this number raises to 66.

19. The data analysis facilities in Melanie II serve different purposes. Displaying spot groups is useful when you want to trace changes in expression of one or a few specific proteins. Differential Analysis and Student's *t*-Test let you automatically highlight spots that are differentially expressed between two set of gels. Finally, Correspondence Analysis automatically analyzes a set of gels, and groups together those that are similar.

20. Results produced by all data analysis algorithms provided in Melanie II have to be interpreted with care. Data analysis is performed on the result of all previously carried out processing. In particular, if gel matching was performed inaccurately, then data analysis may yield completely erroneous results.

21. Double clicking on a spot is the easiest way to create a label, but alternatively, labels may be created with the *Edit → Labels → Add Labels* option. Select one or more features using the Feature tool, and then choose *Edit → Labels → Add Labels*. The label with be added to all selected features.

22. SWISS-2DPAGE masters contain labels for all proteins corresponding to entries in the SWISS-2DPAGE database. Each label holds the accession number (AC) of the database entry, as well as the entry's short name. For example, the Human Plasma protein map possesses a label whose AC is P02768 and name is ALBU_HUMAN for each feature that corresponds to albumin. In addition to normal masters, spots in SWISS-2DPAGE master gels also present the particularity of possessing serial numbers, an identification number that uniquely links a feature on the gel to protein data in the SWISS-2DPAGE database. The serial number has the form 2D-xxxxxx, where x is a digit or an upper-case letter. For example, 2D-000C5W and 2D-000C6F identify two spots on the Human Plasma protein map that have been identified as apo E. The serial numbers are embedded in the SWISS-2DPAGE master gels and may not be changed. Updated SWISS-2DPAGE master gel images may be retrieved from the ExPASy FTP server at ftp.expasy.ch in the directory/databases/swiss-2dpage/masters. The complete URL is: ftp://ftp.expasy.ch/databases/swiss-2dpage/masters

23. SWISS-2DPAGE masters reflect the data contained in the SWISS-2DPAGE database. Because the database is updated on a regular basis, you should from

time to time update your copies of the SWISS-2DPAGE masters by retrieving them from the Expasy FTP server as detailed in **Note 22**.

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41

2-D Databases on the World Wide Web

Ron D. Appel, Amos Bairoch, and Denis F. Hochstrasser

1. Introduction

Many laboratories have identified proteins on two-dimensional (2-D) maps, and have built a master gel and a related 2-D database containing data specific to the identified proteins. Several of these databases have been made available on the World Wide Web (WWW) *(1)*, and many more will undoubtedly follow. This chapter shows how to retrieve data remotely from these databases over the Internet.

2. Materials

2.1. Hardware

1. Computer:
   a. Sun Microsystems® SPARCStation 4 or better, 64 MB of memory, 1 GB of hard disk, and the Solaris 2.4 (or later) operating system.
   b. Apple Power Macintosh®, 32 MB of memory and 1 GB of hard disk.
   c. PC-compatible computer, 32 MB of memory and 1 GB of hard disk, MS Windows NT 4.0 (or later), or MS Windows 95 operating system.
2. Internet:
   a. Connection to Internet.
   b. Internet access, obtainable through your system administrator or any commercial Internet provider.

2.2. Software

   a. Melanie II 2-D PAGE software, Bio-Rad Laboratories, Hercules, CA.
   b. Netscape Navigator®: For academic purposes, a free copy can be downloaded from Internet. Look at the following URL address for further details: http://home.netscape.com/comprod/mirror/index.html.

3. Methods

There are basically two ways of retrieving data from a 2-D database over the Internet: querying and browsing the database using a WWW browser such as Netscape Navigator, or directly linking spot data on a master gel within the Melanie II 2-D PAGE software *(2,3)* to corresponding entries in the remote database. The latter method also requires the Netscape Navigator browser.

3.1. Querying a 2-D Database

2-D databases accessible on the WWW are cataloged in the WORLD-2DPAGE Web page that is located at URL: http://www.expasy.ch/ch2d/2d-index.html. This document is updated on a regular basis. It lists federated 2-D databases, that is, 2-D databases that can be:

1. Remotely queried on the WWW.
2. Attainable through a search in the SWISS-PROT protein sequence database.
3. Linked to other 2-D databases through hypertext links.
4. Queried graphically by clicking on a spot in a 2-D gel image.
5. Directly reached from within 2-D analysis software.

See **ref. *4*** for more details on federated 2-D databases (*see* **Note 1**).

To query a 2-D database over the Internet, run Netscape Navigator, and perform the following operations:

1. Go to WORLD-2DPAGE. Choose Open Location in menu File, and enter the following URL: http://www.expasy.ch/ch2d/2d-index.html. Click OK. The WORLD-2DPAGE document will be retrieved from the ExPASy WWW server.
2. Go to 2-D Database. In the list of federated 2-D database, select the database of your choice, and click on it. The database's home page will be requested from the corresponding Web server *(5,6)*. If you have selected the SWISS-2DPAGE database *(7,8)*, then you may choose among the following options to query the database (*see* **Note 1** for other 2-D databases):
   1. Query by keyword.
   2. Query by accession number.
   3. Query by clicking on a spot.
   4. Query by author.

3.1.1. Query by Keyword

1. Select option. In the section named Access to SWISS-2DPAGE, click on "by description line (DE) or by ID." This requests the keyword search page from ExPASy.
2. Enter keyword. Once the keyword search page is displayed on your screen enter a keyword of your choice in the designated field (*see* **Note 2**). For example, you may enter the term isomerase in order to retrieve all SWISS-2DPAGE entries whose descriptions contain the word isomerase. Click the return key. Netscape will send the query to ExPASy and will then display the result.
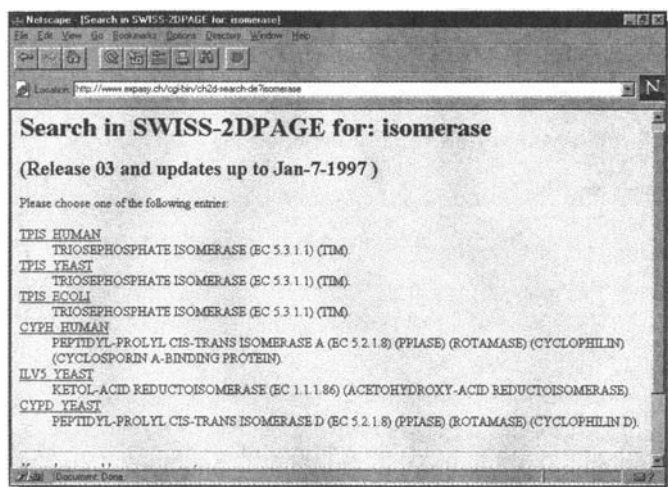
Fig. 1. Entries in SWISS-2DPAGE that match the keyword isomerase.

3. Select database entry. The result page shows all entries in the database that match the queried keyword. The entries are listed by their short name (ID) (**Fig. 1**). Select one of them and click on it. For example, click on TPIS_HUMAN. This will retrieve the complete entry from SWISS-2DPAGE. Various types of information are shown in this document, such as the protein short name (ID), its accession number in the database (AC), its full description (DE) and bibliographical references, as well as active crossreferences to other related databases and active icons to available SWISS-2DPAGE master images (**Figs. 2** and **3**).

4. Compute pI/M_r. To obtain the theoretical pI and M_r of the protein, click on Compute pI/M_w (*see* **Note 3**).

5. Get gel image with spot. To obtain the full-size image of one of the available master protein maps, and to see the exact location of the protein, click on the corresponding icon in the section called 2-D PAGE maps for identified proteins. For example, you may select the HEPG2 map (**Fig. 4**).

6. Get gel image without spot. Just to retrieve the theoretical region in which you may expect to find the protein on any of the master gels the protein has not been identified in, click on the corresponding icon in the section called 2-D PAGE maps for unidentified proteins. For example, click on PLASMA.

7. Access related databases. In the crossreference lines (DR), you may click on any of the links to retrieve corresponding entries, either from the SWISS-PROT protein sequence database *(9)* or from other 2-D databases. From the SWISS-PROT entry, you can then access related data from numerous other databases *(5)*. For example, from the SWISS-2DPAGE TPIS_HUMAN entry, you may directly reach the entry for TPIS_HUMAN in the HSC-2DPAGE database of the Heart Science Centre at Harefield Hospital.
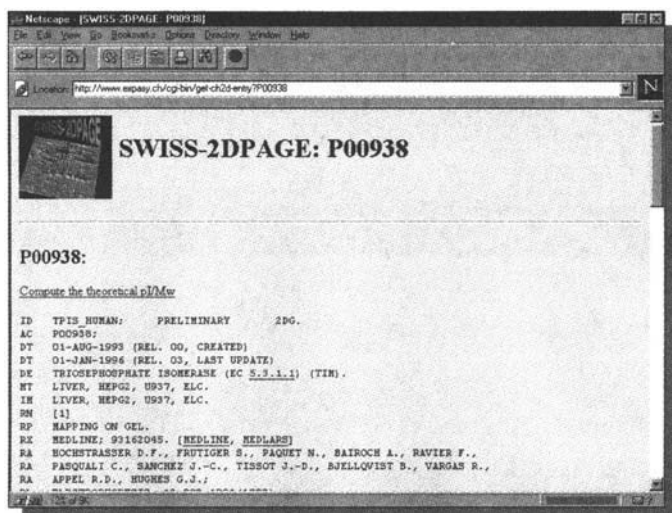
Fig. 2. Top of the TPIS_HUMAN entry in SWISS-2DPAGE (showing the protein ID, AC, description, creation and modification date, as well as bibliographical references).

3.1.2. Query by Accession Number

1. Select option. In the section named Access to SWISS-2DPAGE, click on "by accession number (AC lines)." This requests the search by accession number page from ExPASy.
2. Enter accession number. Once the search by accession number page is displayed on your screen, enter the accession number of your choice in the designated field. For example, you may enter P00938. Click the return key. Netscape will send the query to ExPASy and will then display the TPIS_HUMAN entry.
3. Access related data. Access additional data as outlined above in **Subheading 3.1.1.** in **steps 4–7**.

3.1.3. Query by Clicking on a Spot

This method lets you choose one of the master gel images that are available on the server, display the gel image, and select one protein entry by clicking on the corresponding spot.

1. Select option. In the section named Access to SWISS-2DPAGE, click on "by clicking on a spot." This requests the SWISS-2DPAGE map selection page from ExPASy.
2. Select master map. Once the SWISS-2DPAGE map selection page is displayed on your screen, select the master reference protein map of your choice by clicking on the respective icon. For example, you may click on the *E-Coli* icon in order to retrieve the image of the *Escherichia coli* master gel (**Fig. 5**).
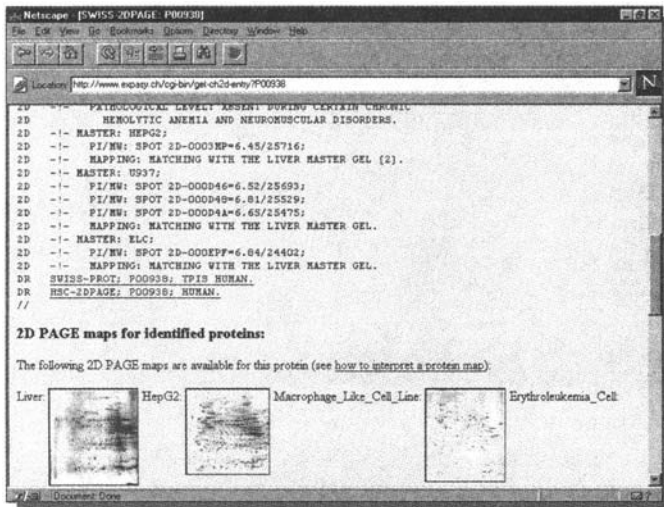
Fig. 3. Second part of TPIS_HUMAN entry in SWISS-2DPAGE (with p*I*, *M*$_r$, and serial number of protein spots in respective master gels, as well as links to SWISS-PROT and to other 2-D databases, and active icons to the full-size master images).
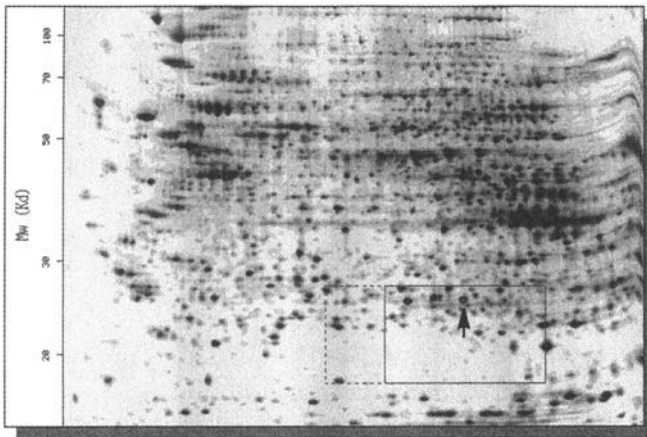
Fig. 4. Area of the HEPG2 master gel, showing location of the TPIS_HUMAN, as well as the theoretical region for the protein, computed from its sequence.

3. Select a spot. On the master image, all identified proteins are marked by a small red cross. Among the marked spots, click on the protein of your choice. The related entry from SWISS-2DPAGE will be requested from ExPASy and displayed on the screen.
4. Access related data. Access additional data as outlined above in **Subheading 3.1.1.** in **steps 4–7**.
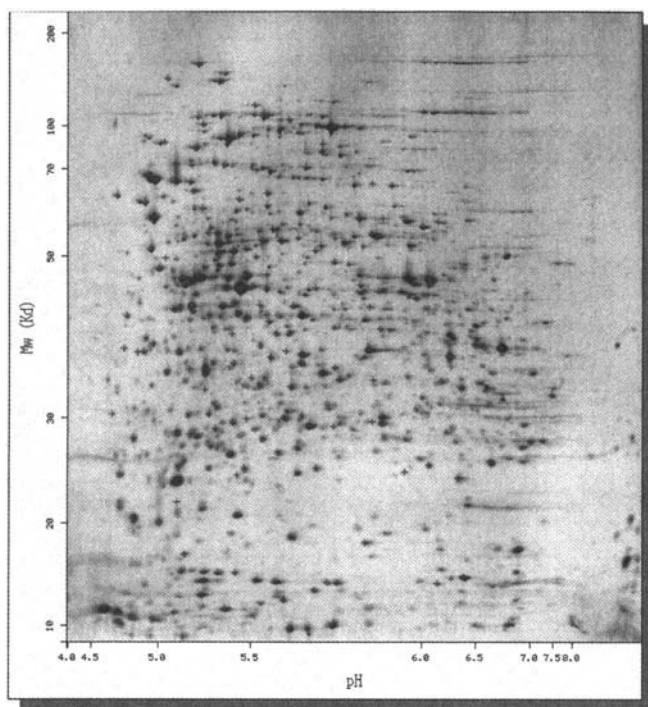
Fig. 5. The *E. coli* master gel with identified proteins marked by a red cross.

3.1.4. Query by Author

1. Select option. In the section named Access to SWISS-2DPAGE, click on "by author (RA lines)." This requests search by author page from ExPASy.
2. Enter author name. Once the search by author page is displayed on your screen, enter a name (or partial name) of your choice in the designated field. For example, you may enter Anderson in order to retrieve all SWISS-2DPAGE entries that reference a paper by Anderson. Click the return key. Netscape will send the query to ExPASy and will then display the result (**Fig. 6**).
3. Select database entry. The result page shows all entries in the database that reference the given author(s). Select one author and one database entry, and click on it. For example, choose Anderson N.G., and click on ACTB_HUMAN. This will retrieve the complete entry from SWISS-2DPAGE.
4. Access related data. Access additional data as outlined in **Subheading 3.1.1.**, **steps 4–7**.

3.2. Linking Spot Data to a 2-D Database

Linking spot data to a 2-D database allows you to retrieve automatically data from a 2-D database while analyzing 2-D gel images using the Melanie II
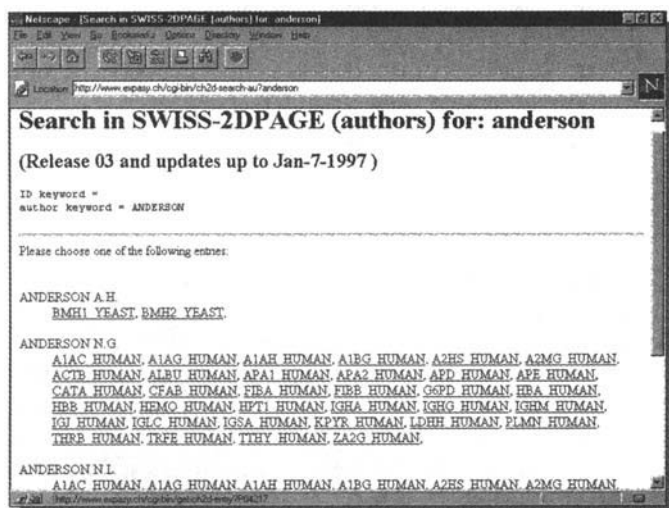
Fig. 6. List of SWISS-2DPAGE entries that contain the word Anderson in their bibliographical references.

2-D PAGE analysis software (*see* **Note 4**). Open or create a master gel, as described in Chapter 40, then follow these steps:

1. Set the master. You have to designate the master gel prior to querying the database. A gel may be master if it contains at least one label with a valid AC (*see* **Note 5**). Take *Database → Set → Master*, and then choose the master from the open gels. Click OK.

2. Setting the browser. You have to set the full path name for Netscape. Choose *Database → Set → Browser*, and enter the program name and path (*see* **Note 6**). The program name alone may be sufficient if the directory in which Netscape resides is part of your path.

3. Setting the server. You can choose the WWW server on which the remote database resides. The server has to host a federated 2-DE database *(4)*. An example of such a WWW server is the ExPASy WWW server. Choose *Database → Set → Server*, and enter the server's host name. For ExPASy (this is the default), enter: www.expasy.ch.

4. Setting the database. You also have to set the name of the remote database. Choose *Database → Set → Database*, and enter the database name. The default is SWISS-2DPAGE.

5. Querying the database. To query the remote database, select one feature (spot) that contains a label with a valid accession number (AC). Choose *Database → Query Server*. This will control the Netscape program on your computer, which will automatically request the corresponding entry from the remote database. For example, if you have set the database to SWISS-2DPAGE on the

www.expasy.ch server, and you select a feature whose label has the accession number P02649, then the command *Database → Query Server* will obtain the entry for Human apo E.

6. Access related data. Access additional data as outlined above in **Subheading 3.1.1.**, in **steps 4–7**.

4. Notes

1. Other 2-D databases may have slightly different interfaces, but all 2-D databases that are federated *(4)* provide a means to query the database by keyword search, to link to other databases, and to access protein data by clicking on a spot on the gel image or directly from a 2-D PAGE analysis software package, such as the one discussed in Chapter 40. There are currently more than 20 databases that are either fully or partially federated.

2. If you search SWISS-2DPAGE by entering more than one keyword, then the search will return entries with both the individual queries. For example, entering isomerase human will search the database for isomerase and human.

3. Protein pI is calculated using pK_a values of amino acids described in refs. *(10,11)* that were defined by examining polypeptide migration between pH 4.5 and 7.3 in an immobilized pH gradient gel environment with 9.2 and 9.8 *M* urea at 15 or 25°C. Prediction of protein pI for highly basic proteins is yet to be studied, and it is possible that current pI predictions may not be adequate for this purpose.

4. For querying a database using a WWW browser as described in **Subheading 3.1.**, the Melanie II software is not necessary. You may access the 2-D databases using just the Netscape Navigator software. Alternatively, you may also use other browsers, such as NCSA's Mosaic or Microsoft Internet Explorer. However, to link spot data to 2-D databases as presented in **Subheading 3.2.**, both Netscape Navigator and Melanie II 2-D PAGE are mandatory.

5. The link between a master gel and 2-D databases is contained in a spot label. As an alternative to creating your own master gel, you may use a SWISS-2DPAGE master. In a SWISS-2DPAGE master gel, each label holds the accession number (AC) of the database entry, as well as the entry's short name. For example, the Human Plasma protein map possesses a label whose AC is P02768 and name is ALBU_HUMAN, for each feature that corresponds to albumin. In addition to normal masters, spots in SWISS-2DPAGE master gels also present the particularity of possessing serial numbers, an identification number that uniquely links a feature on the gel to protein data in the SWISS-2DPAGE database. The serial number has the form 2D-xxxxxx, where x is a digit or an upper-case letter. For example, 2D-000C5W and 2D-000C6F identify two spots on the Human Plasma protein map that have been identified as apo E. The serial numbers are embedded in the SWISS-2DPAGE master gels and may not be changed. The serial numbers are embedded in the SWISS-2DPAGE master gels and may not be changed. Updated SWISS-2DPAGE master gel images may be retrieved from the Expasy FTP server at ftp.expasy.ch in the directory /databases/swiss-2dpage/masters. The complete URL is: ftp://ftp.expasy.ch/databases/swiss-2dpage/masters.

6. Setting the browser is not required on all platforms. For example, on a Macintosh, the path to Netscape is automatically set when you first run Netscape manually. On computers where setting the browser is requested, you may also make the path permanent from the *File → Preferences* menu option.

Acknowledgments

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42

Comparing 2-D Electrophoretic Gels Across Internet Databases

Peter F. Lemkin

1. Introduction

In **ref. *1***, we described a computer-assisted visual method, Flicker, for comparing two two-dimensional (2-D) protein gel images across the Internet is described (*see* **Note 5**). This approach may be useful for comparing similar samples created in different laboratories to help putatively identify or suggest protein spot identification. 2-D gels and associated databases are increasingly appearing on the Internet *(2–13)* in World-Wide Web (WWW or web) *(14)* servers and through federated databases *(15)*. **Table 1** lists some web URL addresses for a number of 2-D protein gel databases that contain 2-D gel images with many identified proteins. This opens up the possibility of comparing one's own experimental 2-D gel image data with gel images of similar biological material from remote Internet databases in other laboratories. This new data analysis method allows scientists to collaborate and compare gel image data over the World Wide Web more easily.

As we suggested, when two 2-D gels are to be compared, simple techniques may not suffice. There are a few ways to compare two images: (1) slide one gel (autoradiograph or stained gel) over the other while back-lighted, or (2) build a 2-D gel quantitative computer database from both gels after scanning and quantitatively analyzing these gels using research *(16–22)* or commercial systems. These methods are impractical for many investigators, since in the first case, the gel from the Internet database is not locally available. The second method may be excessive if only a single visual comparison is needed because of the costs (labor and equipment) of building a multigel database solely to answer the question of whether one spot is the same spot in two gels.

Table 1
Partial List of WWW 2-D Electrophoretic Gel Databases

ExPASy (liver, plasma, CSF, and so forth, SWISS-2DPAGE), http://expasy.hcuge.ch/
Argonne Protein Mapping Group (mouse liver, human breast cell lines), http://
   www.anl.gov/CMB/PMG/
Heart Science Centre, Harefield Hospital (Human Heart 2D gel Protein DB), http://
   www.harefield.nthames.nhs.uk/
Berlin Human Myocardial 2-D Electrophoresis Protein Database, http://www.chemie.
   fu-berlin.de/user/pleiss/
Cambridge 2-D PAGE (rat neuronal database), http://sunspot.bioc.cam.ac.uk/
   NEURON.html
Embryonal Stem Cells (Immunobiology, University of Edinburgh), http://www.
   ed.ac.uk/ simnh/2DPAGE.html
Human Colon Carcinoma Protein Database (Joint Protein Structure Lab), http://
   www.ludwig.edu.au/www/jpsl/jpslhome.html
Keratinocyte database (Danish Centre for Human Genome Research), http://biobase.
   dk/cgi-bin/celis/
CSH QUEST Protein Database (yeast, REF52 rat, mouse embryo), http://siva.cshl.org/
E. coli Gene-Protein Database Project—ECO2DBASE (in NCBI repository) http://
   pcsf.brcf.med.umich.edu/eco2dbase/
Yeast 2D-PAGE, http://yeast-2dpage.gmm.gu.se/
Large-Scale Biology Corp (2-D maps: rat, mouse and human liver), http://www.
   lsbc.com/patterns.htm
PROTEOME Inc (YPD—Yeast Protein Database), http://www.proteome.com/
   YPDhome.html
NIMH-NCI Protein Disease Database (PDD) (plasma, CSF, urine), http://www-pdd.
   ncifcrf.gov/
Itemize

   [a]Individual gel images with identified proteins are available in these databases. The user
should investigate them individually since URL paths for 2D gel image files will differ.

   This distributed Flicker gel comparison program runs on any WWW-con-
nected computer. It is invoked from the user's Java-capable web browser where
it is then loaded from the NCI Flicker web server. One gel image is read from
any Internet 2-D gel database (e.g., SWISS-2DPAGE, and so forth), the other
may reside on the investigator's computer where they were scanned, or the two
gel images may be from either source. Portions of this chapter were derived
from **ref. *1***.

   The Flicker program is written in Java, a general-purpose, object-oriented
programming language developed by Sun Microsystems *(23)*. Java is rapidly
becoming a standard for portable Internet applications using the WWW. Pri-
mary web sites for information on Java are http://java.sun.com/and http://

```
        Paradigm for distributed-data using Internet client-server

  +---- Internet ----+-------------- ~ ---+--- ~ ---+--- ... ---+
     |                |                   |         |           |
     |                |                   |         |           |
user's Web browser  Web server          DB1       DB2         DBn
   | to run          |---> Flicker              Federated
   | Flicker         |       Java program       2D Gel Web
   |                 |---> 2D gel images        Databases
user's gel files     |       in DBfs

  USER'S COMPUTER   NCI WEB SERVER         OTHER WEB IMAGE DATABASES
```
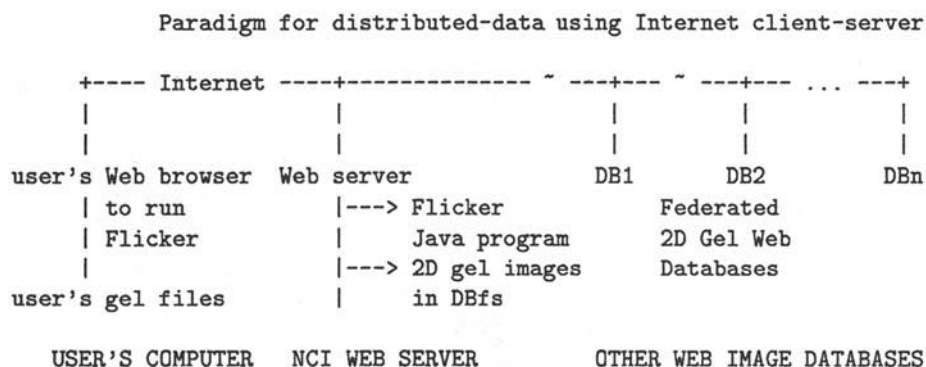
Fig. 1. This illustrates the client–server relationship between the user's web browser with local 2-D gel images, the web server, which contains the Flicker program, and the 2-D gel image web databases (DB) on other web servers. Two gels to be compared may come from the Internet Web databases or from the user's local file system. The images may be from either the Flicker 2-D gel image DB web server DBfs or from other 2-D gel image web databases DB1, DB2,…, DBn. For example, DB1 might be the SWISS-2DPAGE, DB2 might be the Danish Keratinocyte database, DB3 might be the Cambridge heart database, DB4 might be the Argonne breast cell line database, and so forth.

www.javasoft.com/. A Java "applet" is the name Sun uses for a miniapplication that runs inside of a web browser when it automatically loads the applet from the web page containing the URL referencing that applet into the user's web browser. The user's web browser must be a Java-capable web browser (such as Netscape 2.0.1 or Microsoft Internet Explorer 3.0 versions or later). Alternatively, a Java "application" is a program that runs by itself on a user's computer, using the Java interpreter rather than a web browser. In addition, when running Java applications, the java executable program (i.e., Flicker) "class" files need to be copied to the user's computer.

In either case, the user interacts with the NCI Flicker web server, http://www-lecb.ncifcrf.gov/flicker, using the client/server paradigm shown in **Fig. 1**. The Flicker program may be thought of as a client that makes requests of a 2-D gel database web server. Because it runs on the user's computer, Java now gives us the ability to do real-time comparisons of local 2-D gel image data with gel images residing in various remote databases on the Internet. Then, regardless of whether Flicker gets data from that web server, other web servers or locally, it will load two images that can then be compared.

Although the original images may be compared, they may be compared more easily by first applying spatial warping or other image-enhancement transforms. When doing spatial warping, regions of interest are first "landmarked" with several corresponding points in each gel image. Then one gel image is warped

to the geometry of the other. Spatial warping does not change the underlying gray-scale values of the synthesized warped image, which would cause local structural objects to appear and disappear, and thus, new spot artifacts might be created. It samples pixels from the original image to be transformed and places them in the output image according to the geometry of the other input image.

Gels are then compared by flickering them rapidly by alternately displaying them in a third "flicker" display window. Using the mouse, the user may drag one gel image over the other to align corresponding spots visually by matching local morphology.

1.1. Image Flickering

The basic concept of using flickering as a dynamic visualization technique is simple. If two images are perfectly alignable, then one could simply align them by overlaying one over the other and shifting one image until they line up. However, many images, such as 2-D PAGE gels, have "rubber-sheet" distortion (i.e., local translation, rotation, and magnification). This means there is more distortion in some parts of the image than in others. Although it is often impossible to align the two whole images at one time, they may be locally aligned piece-by-piece by matching the morphology of local regions.

If a spot and the surrounding region do match, then one has more confidence that the objects are the same. This putative visual identification is our definition of matching when doing a comparison. Full identification of protein spots requires further work, such as cutting spots out of the gels and subjecting them to sequence analysis, amino acid composition analysis, mass-spectrometry, testing them with monoclonal antibodies (MAbs), or other methods.

1.2. Image Enhancement

It is well known that 2-D gels often suffer from local geometric distortions making perfect overlay impossible. Therefore, making the images locally morphologically similar while preserving their gray-scale data may make them easier to compare. Even when the image subregions are well aligned, it is still sometimes difficult to compare images that are quite different. Enhancing the images using various image transforms before flickering may help. Some of these transforms involve spatial warping, which maps a local region of one image into the geometry of the local region of another image while preserving its gray-scale values. Other transforms include image sharpening and contrast enhancement. Image sharpening is performed using edge-enhancement techniques, such as adding a percentage of the gradient or Laplacian edge detection functions to the original gray-scale image. The gradient and Laplacian have higher values at the edges of objects. Another useful operation is contrast enhancement, which helps when comparing very light or very dark regions by

adjusting the dynamic range of image data to the dynamic range of the computer display. In all cases, the transformed image replaces the image previously displayed. Other functionality is available in Flicker and is described in the Flicker on-line HELP, the **Subheading 3.**, and in **ref. 1**.

1.3. Image-Processing Transforms

As mentioned, there are a number of different image transforms that can be invoked from the control panel.

1.3.1. Affine Spatial Warping Transform

The spatial warping transforms require defining several corresponding landmarks in both gels. As we mentioned, one gel image can be morphologically transformed to the geometry of the other using the affine or other spatial warping transformations. These transforms map the selected image to the geometry of the other image. They do not interpolate the gray scale values of pixels—just their position in the transformed image. As described in **ref. 1**, this might be useful for comparing gels that have some minor distortion, comparing local regions, gels of different sizes, or gels run under slightly different conditions. Flicker uses the affine transform as an inverse mapping as described in **ref. 24**. Let $(u,v) = f(x,y)$, where (x,y) are in the output image, and (u,v) are in the input image. Then, in a raster sweep through the output image, pixels are copied from in the input image to the output image. The affine transformation is given in **Eqs. 1** and **2**.

$$u(x,y) = ax + by + c \tag{1}$$

$$v(x,y) = dx + ey + f \tag{2}$$

When the affine transform is invoked, Flicker solves the system of six linear equations for coefficients (a, b, c, d, e, f) using three corresponding landmarks in each gel.

1.3.2. Pseudo-3-D Transform

As described in **ref. 1**, the pseudo-3-D transform is a forward mapping that generates a pseudo-3-D relief image to enhance overlapping spots with smaller spots seen as side peaks. The gel size is width by height pixels. The gray value determines the amount of shift scaled by a percentage zScale (in the range of 0–50%). Pseudo-perspective is created by shifting the image to the right (left) by angle θ (in the range of –45 to 45°). The transform is given in **Eqs. 3–5**.

$$dx = width \ ^* \sin (\theta), \tag{3}$$

$$x' = [dx * (height - y) / height] + x \tag{4}$$

$$y' = y - z \ scale \ ^* \ g(x,y) \tag{5}$$

where (x,y) is in the original input image and (x',y') is the corresponding position in the output mapped image. Pixels outside of the image are clipped to white. The pseudo-3-D transform is applied to both images so that one can flicker the transformed image.

1.3.3. Edge Sharpening

Edge sharpening may be useful for sharpening the edges of fuzzy spots. The sharpened image function $'(x,y)$ is computed by adding a percentage of a 2-D edge function of the image to original image data (x,y) as shown in **Eq. 6**. The edge function increases at edges of objects in the original image and is computed on a pixel-by-pixel basis. Typical edge functions include the eight-neighbor gradient and Laplacian functions, which are described in **ref. *1*** in more detail. The eScale value (in the range of 0–50%) is used to scale the amount of edge detection value added.

$$g'(x,y) = [e \text{ scale } ^* \text{ edge } (x,y) + (100 - e \text{ scale}) ^* g(x,y)]/100 \qquad (6)$$

2. Materials

The following lists all items necessary for carrying out the technique. Since it is a computer technique, the materials consist of computer hardware, software and an Internet connection. We assume the user has some familiarity with computers and the WWW.

1. A computer with a Java-compatible browser and an Internet connection is required. The actual computer could be a Windows-PC, Macintosh, or Unix X-window system. The computer should have a minimum of 16 Mbytes or more, since intermediate images are held in memory when image transforms occur. If there is not enough memory, it will be unable to load the images, the transforms may crash the program, or other problems may occur. Because a lot of computation is being performed, a computer with at least the power of an Intel 486/66 PC or better is suggested.
2. The Java-compatible browser should be Netscape 2.0.1 or later, or Internet Explorer 3.0 or later.
2'. Alternatively, if you decide to run Flicker locally as a Java application, then you will need the Java interpreter called java. This is available free as part of the Java Development Kit (JDK) over the Internet from SUN or other mirror sites http://java.sun.com/ and http://www.javasoft.com/. Alternatively, it is often packaged on CD-roms in the back of books on Java. If you decide to go this route, you will need to read the documentation that comes with the JDK software on how to install it for your particular machine. The 2' alternative method is trickier to use than method 2, since more computer-specific systems knowledge is required to set it up. You will also need to download the Flicker "class" files from http://www-lecb.ncifcrf.gov/flicker/flicker.zip and put these in a class directory on your computer.
3. You will need a list of specific GIF image URLs from Internet 2-D gel image databases and/or copies of locally scanned gel images in GIF format. You can

use the list of 2-D gel web databases in **Table 1** as a starting point for finding gel GIF images you could download with Flicker. You might investigate the ExPASy 2D-hunt web page, which is a search engine for 2-D gel electrophoresis web sites at http://www.hon.ch/cgi-bin/2DHunt/find.

4. If the investigator will be using his or her own scanned gels, he or she will need either to have the gel scanner on the machine where Flicker will run or arrange to transfer the image files to that machine. In addition, the gel images may need to be converted to GIF images required by Flicker software (e.g., many scanners generate TIFF formatted images). Image format conversion software may be part of your scanner software. If not, there are a number of image file format converters available as part of various desktop publishing packages. Also some converter software is available free from the Internet (use a Web search engine, such as Alta Vista to help find it).

3. Methods

3.1. Installation

We now describe the operation of the Flicker applet from the point of view of the user. You first start up Flicker. This may include the specification of particular images from the NCI Flicker server. Otherwise, you need to specify the gel images to load once Flicker is running. Then you simply flicker the gel images or use image-enhancement transforms first and then flicker them.

3.1.1. Installing Flicker

Installing Flicker under a web browser—nothing is needed (*see* **Note 1**)! Assuming you have a working web browser on your Internet-connected computer, there is nothing to do, since Flicker is automatically downloaded into your browser every time you invoke its URL on the NCI web server: http://www-lecb.ncifcrf.gov/flicker.

For those who are interested in the details on how Flicker is invoked, you can see some of the HTML (HyperText Markup Language) examples of how to start Flicker with prespecified images from HTML in files linked from the Flicker home page, http://www-lecb.ncifcrf.gov/flicker/. These use the HTML <APPLET> and </APPLET> tags. The following is an example of the APPLET HTML code required to start Flicker with two images.

```
<APPLET >
CODEBASE=http://www-lecb.ncifcrf.gov/flicker/
CODE=FlkJ2.class ARCHIVE=FlkJ2.jar
WIDTH=650 HEIGHT=700 ALIGN=absmiddle
ALT="A java-enabled browser is needed to view Flicker applet.">
<PARAM NAME=image1 VALUE= plasmaH.gif>
<PARAM NAME=image2 VALUE= PlasmaL.gif>
</APPLET>
```

3.1.2. Installing Flicker as an Application

If you will be running Flicker as a Java application on your computer, installing Flicker is more complicated. You will use the Java interpreter program called (surprise) java. First, you need to get and install the Sun Java Development Kit (JDK) for your computer. The easiest way is to buy a book on Java for your computer that has a CD-rom with the JDK on it, and then follow the instructions in the book on how to install and use it. Alternatively, you can get it on the Internet from Sun's Java web server http://java.sun.com/.

Next, you need to download the "class" files from the NCI Flicker server using http://www-lecb.ncifcrf.gov/flicker/flicker.zip. The download can be done with Netscape or Internet Explorer (see their documentation) saving the flicker.zip file either in the directory where the images are kept or in some class file directory you create. If the latter is the case, you will need to specify the class path directory to Java using the -classpath option when you run the java interpreter.

Then to run Flicker, type the following command to your computer: java flicker.zip (optional image files or URLS) or java -classpath (your class path) flicker.zip (opt. image files or URLS).

3.2. Graphical User Interface for Flickering

Figure 2 shows the screen of the Flicker applet as seen from a Netscape browser. Control pull-down menus at the top invoke file operations, landmarking, image transforms, and object quantification. Scroll bars on the side determine various parameters used in the transforms. The two images to be compared are loaded into the lower scrollable windows. A flicker window appears in the upper-middle of the screen. Check-boxes on the left activate flickering and control display options. A set of status lines below the check-boxes indicate the state of operations.

Only part of an image is visible in a scrollable window. This subregion is determined by horizontal and vertical scroll bars. Another, preferred method of navigating the scrollable images is to click on the point of interest while the CONTROL key is pressed. This will recenter the scrollable image around that point. This lets the user view any subregion of the image at high resolution. These images may be navigated using either the scroll bars or by moving the mouse with the button pressed in the scrollable image window. Then, each

---

Fig. 2. *(opposite page)* Screen view of Netscape running the Flicker Java applet. Control menus at the top invoke file operations, landmarking, image transforms, and object quantification. Scroll bars on the side determine various parameters used in the transforms. File menu options include loading new images from URLs on the Internet or the local file system, resetting images after a transform, aborting the current transform in progress, and help. Check-boxes on the left activate flickering and control
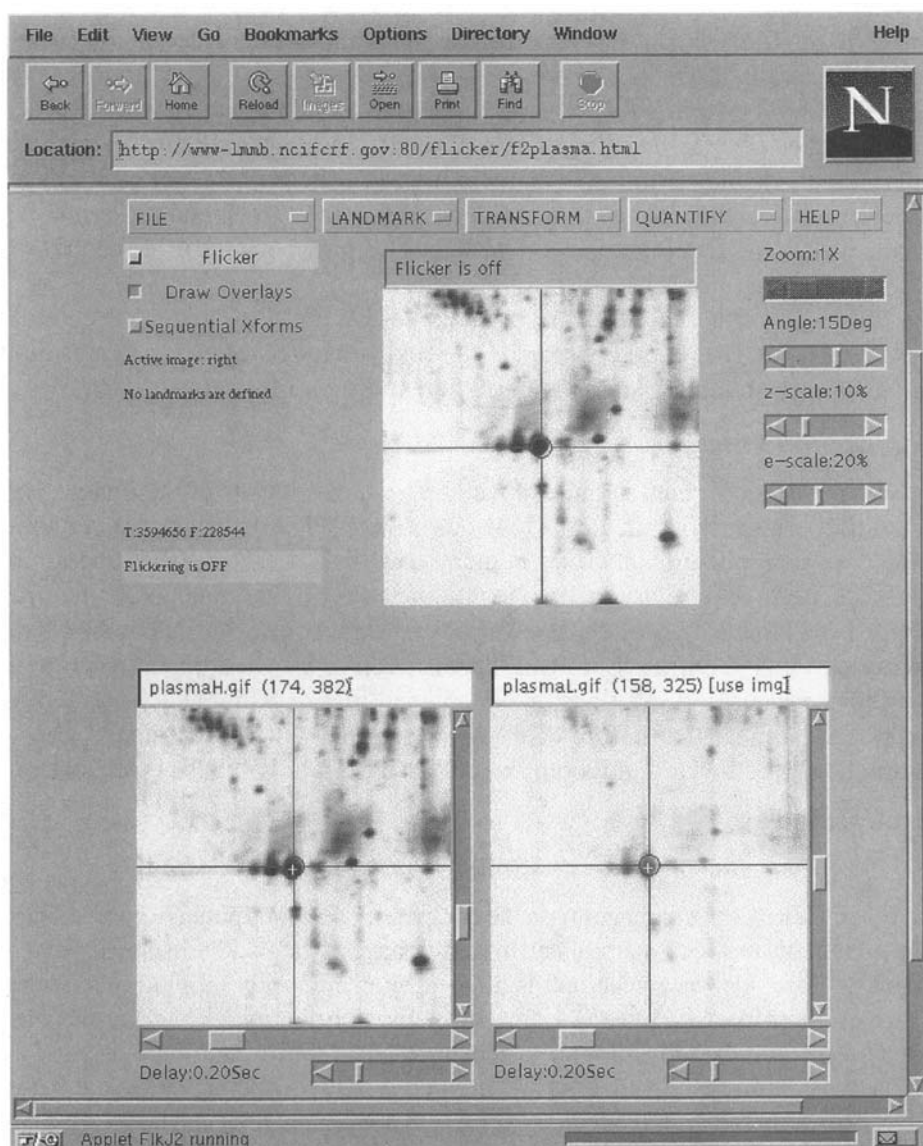
display options. A set of status lines below the check-boxes indicate the state of operations. The flicker image is in the upper-middle of the frame when it is enabled. The two labeled human blood plasma gel images are shown in the bottom scrollable windows, which may be positioned to the region of interest. These windows also have associated flicker time-delays used when flickering. Image plasmaH is an IPG nonlinear gradient gel from Denis Hochstrasser's Lab in Geneva, and plasmaL is a non-IPG linear gradient gel from Carl Merril's Lab at NIMH. Transformed image results are shown in the same scrollable windows.

image in the flicker window is centered at the point last indicated in the corresponding scrollable image window.

A flicker window is activated in the upper-middle of the screen when the "[ ] Flicker" check-box is set. Images from the left and right scrollable images are alternatively displayed in the flicker window. The flicker delay for each image is determined by the setting of the scroll bar below the corresponding scrollable image window. Various graphic overlays may be turned on and off using the "[ ] Overlays" check-box.

Clicking on either the left or right image selects it as the image to use in the next transform. However, clicking on the flicker image window indicates that the transform should be applied to both left and right images.

3.3. Loading Images

When Flicker is running under a web browser, the names of the images are fixed and are specified in the HTML (as shown above). If you are running Flicker as an application, then select the "Load File" or "Load URL" operation in the "FILE" menu to load an image into the left or right image. Note that the image needs to have been selected by clicking on it before you select the FILE menu load image option. Because Flicker is run as a Java application, it will not impose security restrictions on which local files or remote URLs you may request. Also note that before enabling flickering or doing any image transforms, you need to wait until both images have been fully loaded (*see* **Note 2**).

3.4. Flickering

3.4.1. Use of Flicker for Comparing Images

When flickering two images with the computer, one aligns putative corresponding subregions of the two rapidly alternating images. The flicker display overlays the same space on the screen and is aligned by interactively moving one image relative to the other (*see* **Note 3**). Using the mouse, the user initially selects what he or she suspects is the same prominent spot or object in similar morphologic regions in the two gel images. The images are then centered in the flicker window at these spots. When these two local regions come into alignment, they appear to "pulse," and the images fuse together. At this point, differences are more apparent, and it is fairly easy to see which spots or objects correspond, which are different, and how they differ. We have found that the user should be positioned fairly close to the flicker window on the screen to optimize this image-fusion effect.

3.5. Selecting the Proper Time Delays When Flickering

The proper flicker delays, or time each image is displayed on the screen, is critical for the optimal visual integration of image differences. We have also found that optimal flicker rates are dependent on a wide variety of factors,

including amount of distortion, similarity of corresponding subregions, complexity and contrast of each image, individual viewer differences, phosphor decay time of the display, ambient light, distance from the display, and so on. We have found the process of flickering images is easier for some people than for others.

When comparing a light spot in one gel with the putative paired darker spot in the other gel, one may want to linger longer on the lighter spot to make a more positive identification. Because of this, we give the user the ability to set the display times independently for the two images (typically in the range of 0.01–1.0 s with a default of 0.20 s) using separate "Delay" scroll bars located under each image. If the regions are complex and have a lot of variation, longer display times may be useful for both images. Differential flicker delays with one longer than the other are also useful for comparing light and dark sample gels.

3.6. Image-Processing Methods

As mentioned, there are a number of different image transforms that can be invoked from the menus. These are useful for changing the geometry, sharpness, or contrast, making it easier to compare potentially corresponding regions. As we go through the transforms, we will indicate how they may be used. Some affect one image, but some affect both. Flickering is deactivated during image transforms to use most computational power for doing the transforms.

The "TRANSFORM" menu has a number of selections that include warping, gray-scale transforms, and contrast functions. The two warp method selections: "Affine Warp" and "Poly Warp" are performed on only one image (the last one selected by clicking on an image). Unlike the warp transforms, the gray-scale transforms are performed on both images. These include: "Pseudo 3D," "SharpenGradient," "SharpenLaplacian," "Gradient," "Laplacian," and "Average." The contrast functions are "Complement" and "ContrastEnhance."

3.6.1. Landmarks: Trial and Active

The affine transform requires three "active" landmarks to be defined before it can be invoked. A "trial" landmark is defined by clicking on an object's center anywhere in a scrollable image window. This landmark would generally be placed on a spot. Clicking on a spot with or without the CONTROL key pressed still defines it as a trial landmark. After defining the trial landmark in both the left and right windows, selecting the" Add Landmark" option in the" Landmark" menu defines them as the next active landmark pair and identifies them with a red letter label in the two scrollable image windows. Selecting the "Delete Landmark" option deletes the last active landmark pair defined.

3.6.2. Affine Transform

The two warping transforms, affine (*see* **Eqs. 1** and **2**) and polynomial, require three and six landmarks, respectively. Attempting to run the transform
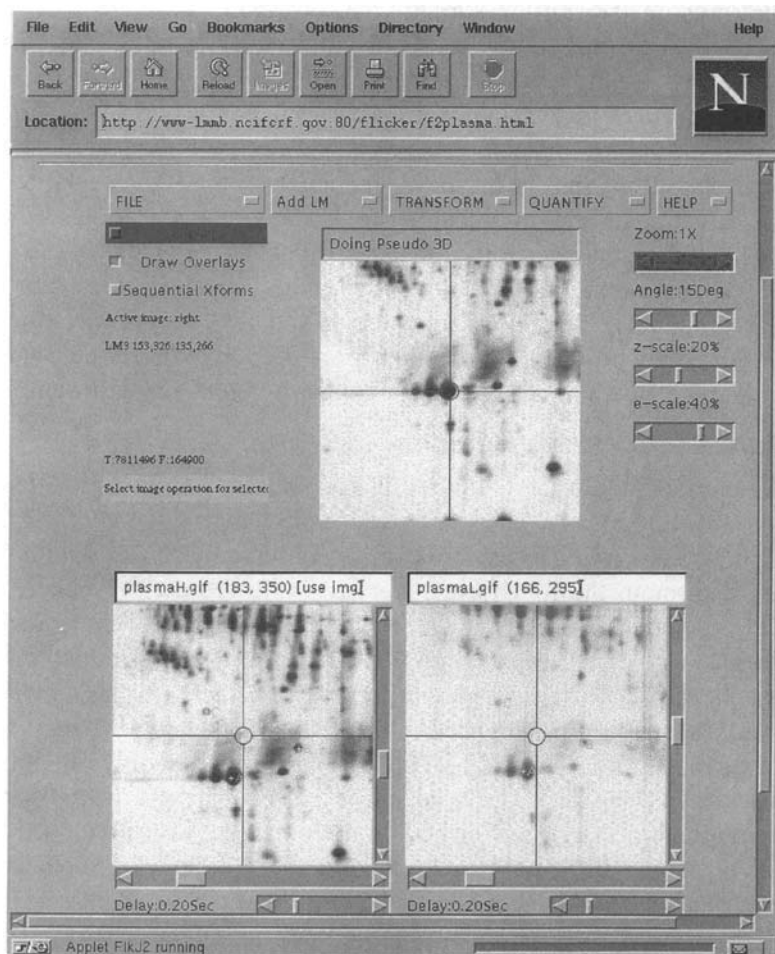
Fig. 3. Screen views of affine transform of human plasma gel image. The transform warps the geometry of a local region defined by the three landmarks, so it more closely resembles the geometry of the corresponding local region in the other gel. **(A)** Scrollable image windows with three "active" landmarks defined in both gel images, which were done in preparation for doing the affine image transform. These corresponding landmark spots were selected to be unambiguously defined in both gel images.

with insufficient landmarks will cause Flicker to notify you that additional landmarks are required. The image to be transformed is the one last selected. You must select either the left or right image. **Figure 3** shows the landmarks the user defined in the two gels before the affine transform. Figure 3B shows the affine transform done on the right gel image.
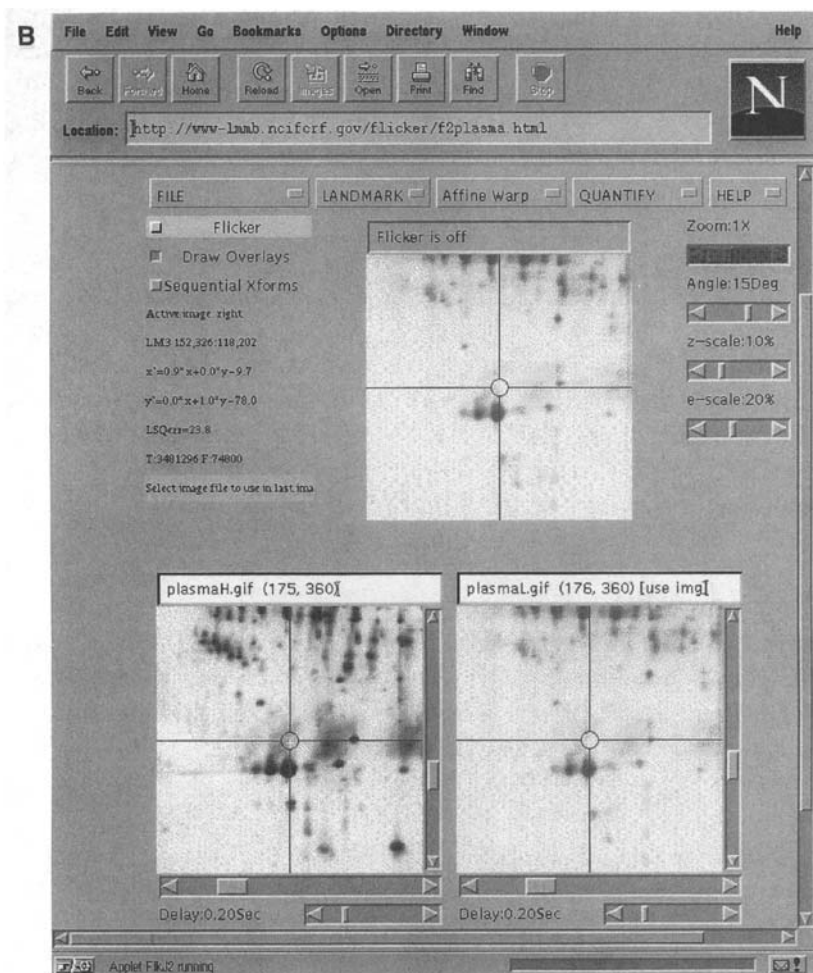
Fig. 3. *(continued)* Screen views of affine transform of human plasma gel image. The transform warps the geometry of a local region defined by the three landmarks, so it more closely resembles the geometry of the corresponding local region in the other gel. **(B)** Scrollable image windows after the affine warp transform of the right (plasmaL, non-IPG) image to the geometry of the left (plasmaH, IPG gel) image.

3.6.3. Pseudo-3-D Transform

As described in **ref. *1*** and as shown in **Eqs. 3–5**, the pseudo-3-D transform generates a pseudo-3-D relief image to enhance overlapping spots with smaller spots seen as side peaks. The gray value determines the amount of shift scaled by a percentage (set by scroll bar z scale) (in the range of 0–50%). Pseudo-

perspective is created by shifting the image to the right (left) set by scroll bar angle θ (in the range of –45 to 45°). The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker-window), then both images are transformed. **Figure 4** shows the results of applying the pseudo-3-D transform to both images.

3.6.4. Edge Sharpening

Edge sharpening may be useful for improving the visibility of the edges of fuzzy spots. You can select either a Gradient or Laplacian edge-sharpening function using the "SharpenGradient " or "SharpenLaplacian" operation in the "TRANSFORM" menu. The image to be transformed is the one last selected. You can set the scroll bar e scale value (in the range of 0–50%) to scale the amount of edge detection value added. The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker-window), then both images are transformed. **Figure 5** shows the results of applying the sharpen Laplacian transform to both images.

3.6.5. Other Image Transforms

There are a number of other image transforms that can be invoked (*see* **Note 4**). Like the edge-sharpening transforms, the image to be transformed is the one last selected, and if neither was selected (i.e., you clicked on the flicker-window), then both images are transformed.

4. Notes

1. There are several advantages of dynamically downloading Flicker each time it is used. Software updates are completely invisible to users, since they do not have to waste time or space installing them on their computers. The technique uses existing low-cost web browser technology, which requires little user effort. In addition, it saves time over the alternative ways that scientists might use to compare 2-D gels and other data.

2. There are several problems with using Java with current web browsers because of restrictions owing to applet security concerns. Because of fears of security breaches, Netscape and other web browser providers have disabled Java applets running on their browsers from reading or writing local files. They also restrict access to web URLs other than the host computer where the Java applet originated (i.e., in this case, the NCI Flicker web server). Unfortunately, this prevents the Flicker applet from loading your local image files or other 2-D gel image databases, not the Flicker web server. It thus prevents you from comparing data from different sources. However, there are two ways to get around the security problem: (1) use the stand-alone Java "appletviewer" with Flicker, or (2) run Flicker as a stand-alone java application using the "java" interpreter. It is expected that the current restrictions will be removed from future web browsers when the security issues are resolved by Sun and Netscape.
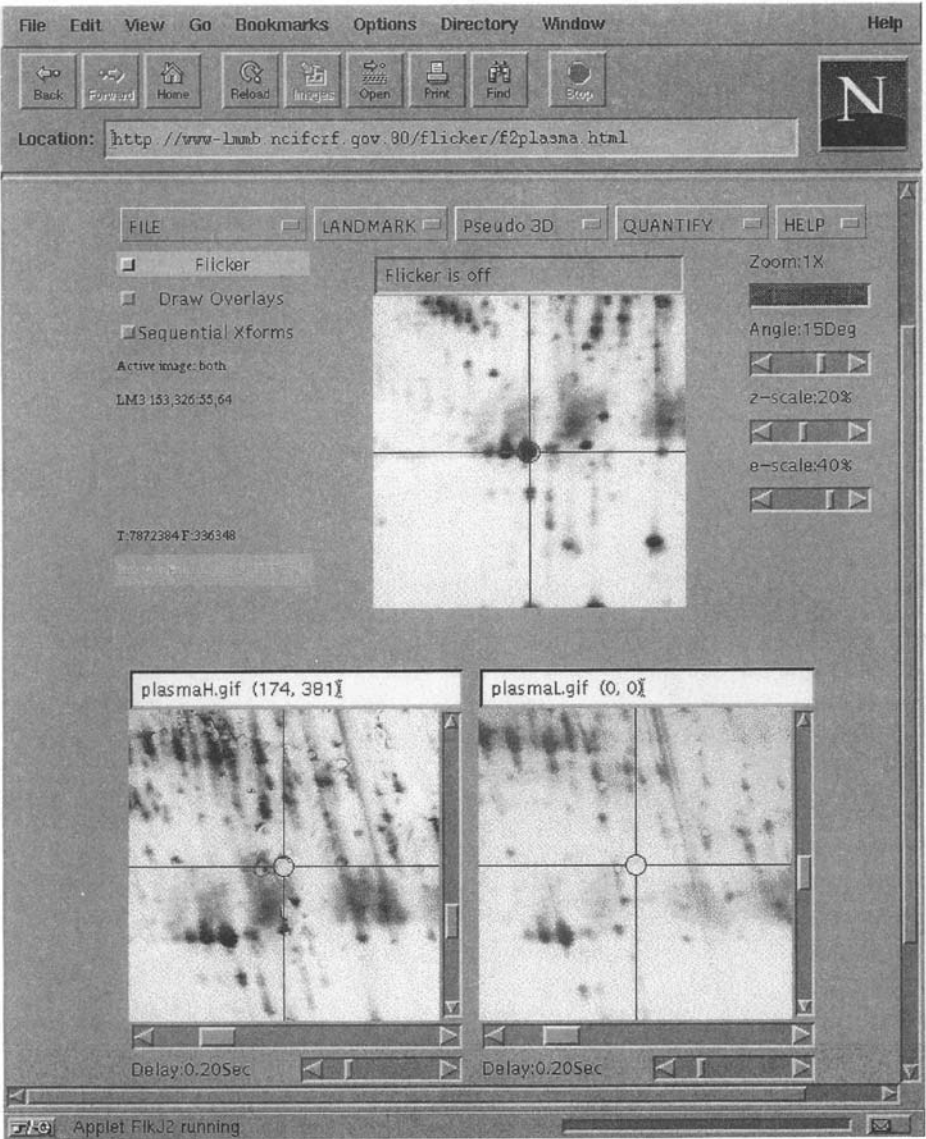
Fig. 4. Human plasma gel images after the pseudo-3-D transform were applied to both gel images. The parameter settings for angle and z scale were 5° and 20%, respectively.

There are several other restrictions. Java currently only handles GIF and JPEG image formats. Images in other formats, such as TIFF, currently need to be converted to GIF format. Because our group is doing image pixel processing with the Flicker program, it requires more memory for intermediate images than programs
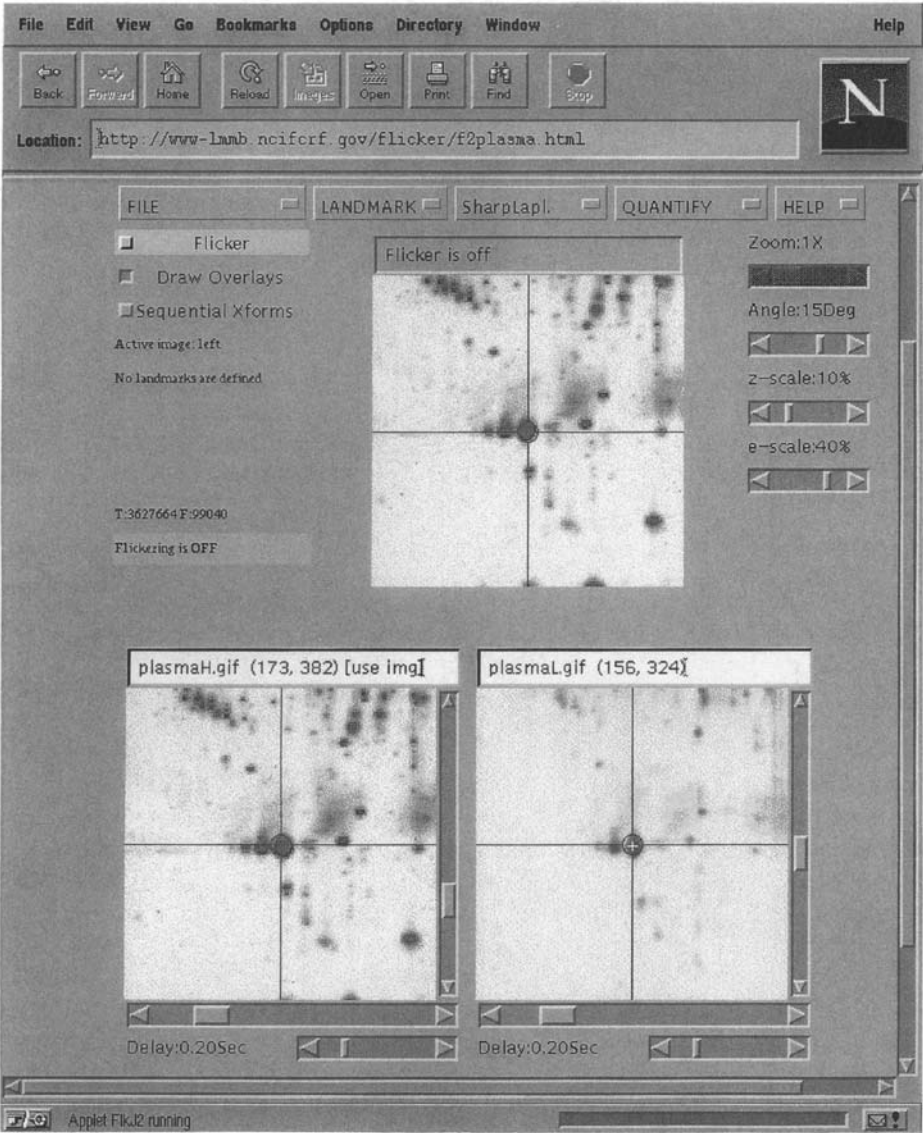
Fig. 5. Human plasma gel images after the SharpenLaplacian transform was applied to both gel images. The parameter settings for e scale was 40%.

that only manipulate text, and so may require a more powerful CPU with more memory than some users currently have. For these reasons, a fast CPU is desirable.

3. However, there are also disadvantages to comparing gels this way. It is only good for doing a rough comparison, and there is currently no simple way available to

do quantitative comparison (as can be done with existing 2-D gel computer database systems *[16–22]*)—although our group is working on the latter. One should keep these limitations in mind when using the technique.

The intent of applying image transforms is to make it easier to compare regions having similar local morphologies, but with some different objects within these regions. Image warping prior to flickering is intended to warp and rescale spatially one image to the "shape" of the other image, so that we can compare them at the same scale. This should help make flickering of some local regions on quite different gels somewhat easier. Of the two warping transforms, affine and polynomial, the latter method handles nonlinearities better. For those cases where the gels are similar, the user may be able to get away with using the simpler (affine) transform.

In cases where there is a major difference in the darkness or lightness of gels, or where one gel has a dark spot and the other a very faint corresponding spot, it may be difficult to visualize the light spot. By differentially setting the flicker display-time delays, the user can concentrate on the light spot using the brief flash of the dark spot to indicate where he or she should look for the light spot. Differential-flicker has been found to be very helpful for deciding difficult cases. Changing image brightness and contrast also is useful when flickering, and the Flicker program has provision for interactively changing these parameters as well.

4. Other transforms, including image sharpening, may be useful in cases where spots are very fuzzy, as might be the case when comparing Southern blots. When two corresponding local regions of the two images are radically different so the local morphologies are not even slightly similar (e.g., when high-mol-wt regions of gels are run differently, such as, IPG vs non-IPG, gradient vs non-gradient SDS), then even using these transforms may not help that much.

5. Of the features and operations we have mentioned, some are not fully functional, and we are working to resolve this. The current state of Flicker is documented in the Flicker Reference Manual http://www-lecb.ncifcrf.gov/flicker/flkInfo.html.

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43

Constructing a 2-D Database for the World Wide Web

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1. Introduction

A federated two-dimensional (2-D) database is a database containing 2-D data that are accessible on the World Wide Web (WWW) and that are dynamically linked to other similar databases through active hypertext links over the Internet *(1,2)*. Federated 2-D databases respect five rules that can be found at URL: http://www.expasy.ch/ch2d/fed-rules.html.

These rules guarantee that a 2-D database may be:

1. Remotely queried on the WWW.
2. Attainable through a search in the SWISS-PROT protein sequence database *(3)*.
3. Linked to other 2-D databases through hypertext links.
4. Queried graphically by clicking on a spot in a 2-D gel image.
5. Directly reached from within a 2-D analysis software.

Existing federated 2-D databases are listed on a WWW page at URL: http://www.expasy.ch/ch2d/2d-index.html.

This chapter demonstrates how to prepare the files necessary to build a federated 2-D database in order to make it available on your own WWW server.

2. Materials
2.1. Hardware

1. Computer: Sun Microsystems® SPARCStation 4 or better, 64 megabytes of memory, 1 gigabyte of hard disk, and the Solaris 2.4 (or later) operating system (*see* **Note 1**).
2. Internet:
    a. Connection to Internet.
    b. Permanent Internet access, obtainable through your system administrator or a commercial Internet provider.

2.2. Software

1. Melanie II 2-D PAGE software, Bio-Rad Laboratories (Hercules, CA).
2. Apache HTTPD Web server daemon, version 1.1 (or later) obtainable at URL: http://www.apache.org/.
3. Perl release five interpreter, retrievable at URL: http://www.perl.com/perl/.
4. The make2ddb package. This software package is available through the URL: http://www.expasy.ch/ch2d/make2ddb.html. This package lets you easily create a 2-D database on your Web server by generating the necessary programs and index files.
5. Netscape Navigator®: For academic purposes, a free copy can be downloaded from Internet. Look at the following URL address for further details: http://home.netscape.com/comprod/mirror/index.html.

3. Methods

To create a 2-D database, you need at least one master gel image built according to the procedure sketched in Chapter 40. The following operations have to be carried out:

1. Install the make2ddb package. During installation, you have to provide certain information (*see* **Note 2**), including:
   a. The path to your server's bin, cgi-bin, and htdocs directories.
   b. The path to the directory in which you place all your 2-D database files; let us call this directory *2d-directory*.
2. Create master. Create a master gel according to the procedure described in Chapter 40 (*see* **Note 3**). The master has to contain at least one label with at least the accession number set (AC). Let us call this master gel file *Master*. Place *Master* in the *2d-directory* directory.
3. Create database text file. For each identified protein in *Master*, that is, for each set of labels with the same AC, you have to create a corresponding text entry. All these entries have to be concatenated, that is, joined together into one big ASCII file of the form xxx.dat. For example, let us call our text file *db.dat*. Each entry should be stored in the SWISS-2DPAGE format *(4)* (*see* **Notes 4** and **5**). Here is an example entry for an *alpha-1 antitrypsin* protein identified in both a plasma and a liver master gel:
   ```
   ID    A1AT_HUMAN; STANDARD 2DG.
   AC    P01009;
   DT    01-AUG-1993 (REL. 01, CREATED)
   DT    01-JAN-1997 (REL. 53, LAST UPDATE)
   DE    ALPHA-1-ANTITRYPSIN (ALPHA-1 PROTEASE INHIBITOR)
           (ALPHA-1-
   DE    ANTIPROTEINASE).
   MT    LIVER, PLASMA.
   IM    LIVER, PLASMA.
   RN    [1]
   ```

```
RP    MAPPING ON GEL.
RX    MEDLINE; 96666666. [MEDLINE, MEDLARS]
RA    DUPOND D.F., DUPONT S., SMITT N., JOHNS A., VANRUPP F.,
RA    JOHNS C., MCDONALD J.-C.;
RL    J. OF ELECTROPHORESIS SCIENCES, 13:992-1001(1996).
RN    [2]
RP    MAPPING ON GEL.
RA    DUPOND D.F., DUPONT S., SMITT N., JOHNS A.;
RL    ELECTROPHORESIS TODAY, 53:707-714(1991).
CC    -!- SUBUNIT: SINGLE CHAIN.
2D    -!- MASTER: LIVER;
2D    -!- PI/MW=4.92/56356;
2D    -!- PI/MW=4.97/55726;
2D    -!- PI/MW=5.01/54641;
2D    -!- MAPPING: MATCHING WITH A PLASMA GEL [1];
2D    -!- MASTER: PLASMA;
2D    -!- PI/MW=5.01/109618;
2D    -!- MAPPING: MICROSEQUENCING [2];
2D    -!- NORMAL LEVEL: 1900-3500 MG/L;
2D    -!- PATHOLOGICAL LEVEL: INCREASED DURING THE
         ACUTE-PHASE
2D    REACTION [2];
DR    SWISS-PROT; P01009; A1AT_HUMAN.
DR    SWISS-2DPAGE; P01009; A1AT_HUMAN.
DR    HSC-2DPAGE; P01009; HUMAN.
```

4. Add header to *db.dat*. Once all entries have been put together, one after the other and separated by the "//" line, into the *db.dat* file, add the following three lines to the top of the file:

   DB-NAME Release NN
   MONTH YEAR

   The first line contains the database name, followed by the word Release and the release number. The second line contains the month and year of the release. The third line is empty. An example heading for db.dat is:

   MY-2DPAGE Release 53
   January 1997

   Place the *db.dat* file in the *2d-directory* directory (*see* **Note 6**).

5. Create GIF images. Using the mastergif and melch2dtogif programs provided with the make2ddb package, create, for each of your masters, the following five images in GIF format, and store them in their respective directories. You have to execute each of these programs in *db-directory*:

   a. melch2dtogif –x -e -l1 -z 0.1 *Master* NULL > *Master*.gif: Place resulting GIF file (*Master*.gif) in directory *2d-directory*/small-gifs/*Master* where *2d-directory* is your database main directory, and *Master* is the name of your master gel image. This file contains the iconified master image.

    b. mastergif -z1.0 -l1 -e *Master*; mv *Master*_id.gif *Master*_id_big.gif.

    c. mastergif -z0.6 -l1 -e *Master*.

    d. melch2dtogif -e -l1 -z1.0 *Master* NULL > *Master*_big.gif.

    e. melch2dtogif -e -l1 -z0.6 *Master* NULL > *Master*.gif.

    For the previous four operations, place resulting GIF files (*Master*.gif, *Master*_big.gif, *Master*_id.gif, *Master*_id_big.gif) in directory *2d-directory*/gifs/*Master*. These files contain the master image with and without red crosses marking the identified proteins, and in large and very large formats respectively.

  6. Create file existing.maps. Create a subdirectory called *2d-directory*/current_release, and place into it a file called existing.maps that contains the list of all your master gel files associated with their respective full names. The first line should contain the word MAPS and the words should be separated by tabulators. An example existing.maps file is:

    MAPS
    LIVER   Human_Liver
    PLASMA   Human_Plasma

    If the full name contains more than one word, then these should be linked by an underscore ("_").

  7. Modify home page. Modify the 2d.html home page provided in the make2ddb package to suit your particular needs, and place the file into the directory htdocs/2d, where htdocs is the directory where you hold your HTML files (*see* **Note 7**).

  8. Add alias to 2d. In your server's configuration directory, edit the file srm.conf in order to add an alias to the htdocs/2d directory. Add a line of the form:

    Alias /2d xxx/2d/

    where xxx is the path to the htdocs directory. For example, add the following line:

    Alias /2d/ /work/www/htdocs/2d/

    This will guarantee that requests to your servers of the form /2d/*.html will find the right directory. For example, if your server's hostname is my.server.edu, then the URL: http://my.server.edu/2d/2d.html will find your database home page.

  9. Run make2ddb. This will create one separate file for each database entry on the *2d-directory*/current_release directory, generate indexes for the keyword and author search, and place correct versions of the cgi-bin programs in your cgi-bin directory. This program will ask you for the name of the *db.dat* file that should be located in *2d-directory*.

 10. Test your 2-D database. Test your database by accessing it through a WWW browser (*see* **Note 8**).

4. Notes

  1. A federated 2-D database may be built and installed on any computer platform, including Unix, Windows, and Macintosh. We have only explained how to install such a database on a Sun under the Solaris operating system, since the make2ddb package available under this architecture greatly simplifies this task.

  2. In order for the various programs to runs correctly, you have to verify that the LD_LIBRARY_PATH environment variable is set to: /usr/openwin/lib:/usr/dt/

lib:/usr/lib. One way to achieve this is to add the following line to the .cshrc or .profile file in your home directory:

setenv LD_LIBRARY_PATH /usr/openwin/lib:/usr/dt/lib:/usr/lib.

Also, you have to check that the following files exist:

/usr/openwin/lib/libXmu.so.4
/usr/dt/lib/libXm.so.3
/usr/openwin/lib/libXt.so.4
/usr/openwin/lib/libX11.so.4
/usr/lib/libC.so.5
/usr/lib/libw.so.1
/usr/lib/libc.so.1
/usr/openwin/lib/libXext.so.0
/usr/lib/libm.so.1
/usr/lib/libsocket.so.1
/usr/lib/libnsl.so.1
/usr/lib/libdl.so.1
/usr/lib/libintl.so.1
/usr/lib/libmp.so.1

3. A master gel is a gel on which proteins have been identified. As in a Melanie II gel, identified proteins are represented by the accession numbers (AC) contained in the labels. For a gel to be a master, you need at least one label with one AC, though a respectable and useful database should obviously contain more such labels.

4. The format of the textual database file does not have to comply precisely with the SWISS-2DPAGE format as outlined in **ref. 4**. Nevertheless, the following lines must absolutely respect this format: ID (identification), AC (accession number), DE (description), MT (master—this gives the list of master samples), IM (image masters—the list of master gel images on which proteins have been identified; this list may, but does not have to match the MT list, since there may be one or more images for one given sample), DR (crossreferences, at least to SWISS-PROT), and // (the last line of each entry containing just the two "/" characters). *See* the example or **ref. 4** for the exact format.

5. The accession number (AC) and ID should be the same as the corresponding protein in SWISS-PROT.

6. The db.dat file's header should match the information in the DT lines. That is, if you produce release 53 of MY-2DPAGE database in January 1997 and if you change any data in the A1AT_HUMAN entry of that release, the db.dat header should show:

MY-2DPAGE Release 53
January 1997

and the first lines of the A1AT_HUMAN entry should indicate (assuming the entry was first created in August 1993):

ID   A1AT_HUMAN; STANDARD 2DG.
AC   P01009;
DT   01-AUG-1993 (REL. 01, CREATED)
DT   01-JAN-1997 (REL. 53, LAST UPDATE)

7. When you update your database, that is, when you either change one or several master gels, or the textual entry file db.dat, execute again the program make2ddb, as well as the mastergif program, as described in **Subheading 3.**, **step 5**.

8. The make2ddb package is under constant development and, thus, may change with time. New versions are available through the URL: http://www.expasy.ch/ch2d/make2ddb.html, and may show small differences compared to what is described here. In case of problems, contact the authors.

Acknowledgments

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Absolute Quantitation of 2-D Protein Spots

Steven P. Gygi and Ruedi Aebersold

1. Introduction

Proteome analysis has been revolutionized by the marriage of two-dimensional (2-D) gel electrophoresis and mass spectrometry *(1–4)*. Mass spectrometry in combination with database searching permits the large-scale identification of proteins, and 2-D gel electrophoresis allows for the large-scale visualization and separation of cellular proteins. Previously, spot quantitation has been difficult, and numbers obtained were usually comparative, but not absolute *(5–7)*. Mass spectrometry and 2-D gel electrophoresis in tandem can be used to quantitate protein spots. This chapter covers protocols to quantitate precisely the relative amounts of protein observed in the 2-D gels using radiolabeled total cell extracts.

The absolute quantitation of protein spots requires that the specific activity (i.e., cpm/pmol protein/methionine) for the cell lysate be calculated. This is done by measuring the protein abundance by amino acid analysis (AAA) for a few known spots (*see* Chapter 47). Next, the radioactivity of these AAA-quantitated spots is counted. Because the number of methionines in each of the selected spots is known, this information combines to give a specific activity (cpm/pmol protein/methionine) for each of the small number of selected spots. This mean value for this number can then be used to quantitate other spots of lesser intensity by knowing the counts present in the spot and the identity of the protein. An overview of the method is shown in **Fig. 1**.

This method has some limitations. For example, the method fails completely for proteins that contain no methionine. In addition, the method requires that the entire sequence be known. This is not a problem for yeast and microbes where the entire genome has been sequenced, but may represents a problem for other organisms. *N*-terminal processing of methionine plays an important role in the functionality of the mature protein, and the cleavage specificity of initiator methionine can often be predicted based on the penultimate amino acid *(8–10)*.
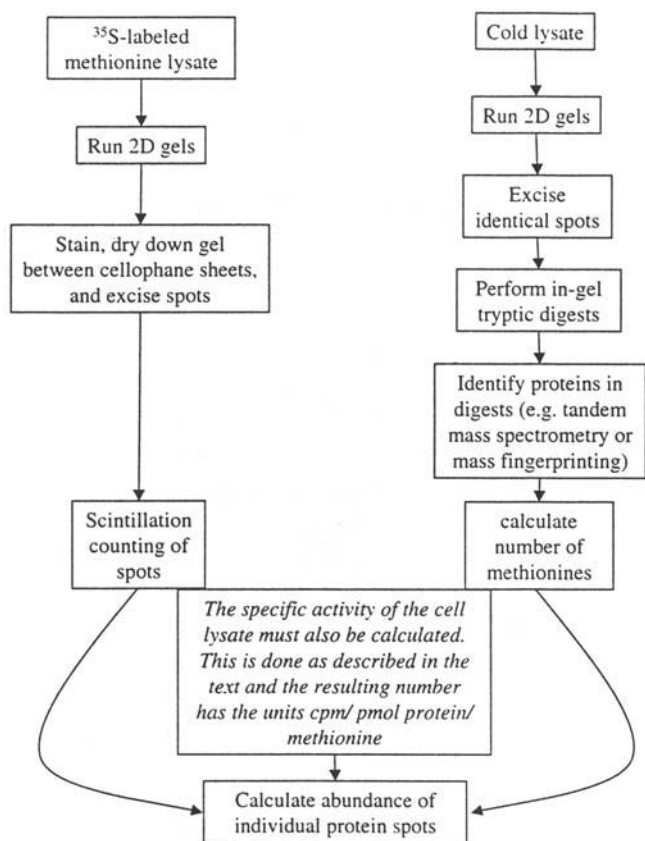
Fig. 1. Flowchart of a process to quantitate and identify proteins observed from a total cell extract.

The half-life of the protein is also important for protein quantitation, and labeling should be done at equilibrium.

2. Materials

1. Cellophane sheets.
2. Gel-drying frame.
3. Scintillation counter.
4. The reagent is scintillation fluid.

3. Methods

3.1. Quantifying 2-D Spots by Radioisotope Incorporation

1. Depending on the organism, prepare a ^{35}S-methionine-labeled total cell extract as described in earlier chapters of this volume (*see* **Note 1**).

2. Using the extract, run two 2-D gels as described in earlier chapters (*see* **Note 2**).
3. Silver-stain one of the gels as described in Chapter 33. Carefully sandwich the wet gel between two pieces of cellophane, and dry the gel using a gel-drying frame (*see* **Note 3**).
4. Transfer the second 2-D gel to a PVDF membrane, and Coomassie stain the membrane as described in Chapters 35 and 36.
5. Using a sharp single-edge razor blade, cut out three to five identical spots from the dried silver-stained gel and from the membrane. The identities and protein sequences of the spots must be known, and the 2-D spots must be well resolved (*see* **Note 4**). To measure the background, cut out at least two unstained pieces of gel as controls (*see* **Note 5**).
6. Place the identical dried gel spots in 0.6-mL microcentrifuge tubes.
7. Add 50 µL of scintillation fluid to the tubes and vortex.
8. Place the tubes in a larger counting vial, and count using a scintillation counter and a ^{35}S protocol (*see* **Note 6**).
9. Perform AAA on the spots from the PVDF membrane to quantitate the amount of protein in each spot (*see* Chapter 47).
10. Calculate the number of methionines for each of the three to five spots for which AAA was performed (*see* **Note 7**).
11. Calculate the mean specific activity of the lysate. A specific activity for each individual protein with the units of cpm/pmol protein/methionine is calculated for each spot and the average taken. The error between the three to five calculations is typically <5%.
12. From the dried gel, cut out all the 2-D spots of interest, and count the radioactivity as described in **steps 7–9**. The identity and protein sequence of these spots must be known.
13. Using the mean specific activity from **step 11**, calculate the abundance of any other 2-D protein spot in the gel (*see* **Note 8**).

4. Notes

1. To determine that the labeling is done at equilibrium, a time-course of incorporation should be taken. Harvest the cells at different labeling times (i.e., 30 min, 2 h, and 6 h). Run the same quantity of each lysate on a 2-D gel, and cut out and count spots of interest at different time-points. If labeling is an equilibrium, the counts will not change over the time-course.
2. We typically load 3×10^6 TCA-precipitable cpm on the gel.
3. This gel is later used for the quantitation of large numbers of identified 2-D proteins by measuring the radioactivity of the 2-D spots. We have observed that if 3×10^6 cpm of a whole lysate are loaded on the gel, then most spots visible by staining can be counted and quantitated. Even the faintest stained spots will produce at least three times the background level of radioactivity.
4. The spots should be well resolved. If two proteins are present in the same sample, they will contribute to errors for both the AAA and radioactivity measurement.
5. The background must be measured and subsequently subtracted from all values prior to any calculation.

6. The counting of spots is straightforward, but can become complicated. The spots should be well resolved. If two spots are present in the same sample, they will contribute to error. In the worst case, a methionine-rich, comigrating protein present at much less abundance than the protein of interest could influence the quantitation of an abundant protein with only a single methionine.

7. The number of methionines in a mature protein should take into account the potential cleavage of the initiator methionine or signal peptide sequences.

8. An example of the calculation is as follows: If the mean specific activity for the cell lysate was found to be 500 cpm/pmol protein/methionine, and a 2-D protein spot containing 6 methionines and 1145 cpm is counted, then 1145 cpm divided by 6 methionines is 191 cpm/methionine. 191 cpm/methionine × 1.0 pmol of protein/500 cpm/methionine is 0.382 pmol protein in the spot. If 1.0×10^6 cells were represented in the lysate, then 0.382 pmol protein/1.0×10^6 cells is 3.82 × 10^{-7} pmol protein/cell or 230,000 copies/cell.

Acknowledgments

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45

Generating a Bacterial Genome Inventory

*Identifying 2-D Spots by Comigrating Products
of the Genome on 2-D Gels*

Ruth A. VanBogelen

1. Introduction

Bacterial genomes range greatly in size, encoding from as few as 500 to more than 4000 proteins. The tools of bioinformatics have become more and more accurate at predicting open reading frames within stretches of DNA, and within this decade, the proteome complement of one or more genomes should be completed. To accomplish such a task, methods for detecting and building an inventory of the protein products encoded by the genome must be used. Such methods have been under way for over 20 years, even before the projects to complete the DNA sequence of the bacterial genome were initiated. Using two-dimensional polyacrylamide gel electrophoresis (2-D gels) *(1)*, one can easily detect the proteins bacteria expressed at levels >50 mol/cell. For the bacterium *Escherichia coli*, about 1200 proteins can readily be detected under any one growth condition.

Many methods to identify proteins separated on 2-D gels have been used. Other chapters in this book discuss methods to identify proteins directly from 2-D gels (or from blots onto support membranes), such as N-terminal sequencing and mass spectrometry. This chapter will describe methods to identify proteins by other means including those that involve:

1. Purified proteins as markers.
2. Growth conditions known to induce or repress certain enzymes.
3. The pattern of peptide fragments.
4. Methods to label protein selectively.
5. Expression of genes cloned into plasmids.

2. Materials

1. Most of the materials needed to grow and radiolabel *E. coli* cells are described in Chapter 3 in this volume along with methods for preparing 2-D protein extracts.
2. Radioactive marker: radioactive ink is made by adding isotope to the replaceable cartridge of an ink pen. Any chemical form of the ^{35}S can be used and is compatible with the disposal of gels containing ^{35}S-labeled proteins. For use on gels containing proteins labeled with isotopes with long half-lives, ^{14}C (any chemical form) should be used.
3. Lysis buffer: 9.9 *M* urea, 4% NP40, 0.1 *M* DTT, 2.2% ampholine mix. Mix together and put at 37°C to get the urea in solution. Store 1 mL aliquots at –80°C.
4. Soaking buffer: 0.125 *M* Tris-HCl, pH 6.8, 1 m*M* EDTA, 0.1% SDS.
5. Overlay buffer: 0.125 *M* Tris-HCl, pH 6.8, 1 m*M* EDTA, 0.1% SDS, 20% glycerol.
6. Proteases solution: 10% glycerol, 0.125 *M* Tris-HCl, pH 6.8, 1 m*M* EDTA, 0.1% SDS.
7. Coomassie blue fixative solution: 50% ethanol, 10% acetic acid, 1% TCA, 0.015% Coomassie blue.
8. Coomassie Blue stain solution: 10% acetic acid, 1% TCA, 0.015% Coomassie blue.
9. Coomassie destain: 7.5% acetic acid.

3. Methods

3.1. General Comments

An accurate estimate of the p*I* and mol-wt-ranges throughout the entire 2-D gel is useful for determining candidate proteins for spots seen on a 2-D gel. Calibrated 2-D gels are used for determining the region on the 2-D gel to which the product of a given gene is expected to migrate *(2,3)*.

3.2. Purified Proteins as Markers

The use of purified proteins for the identification of a gene product or previously characterized protein is fast and efficient method of identification, and requires <10 µg of purified protein.

1. If the purified proteins is lyophilized, resuspend the protein in lysis buffer to a final concentration of 0.5 mg/mL (*see* **Note 1**).
2. Run five 2-D gels with the following loading onto the first-dimension gel (*see* **Note 2**):
   a. Gel 1—1 µg of purified protein only.
   b. Gel 2—0.1 µg of purified protein only.
   c. Gel 3—1 µg of protein plus one million cpm of cellular extract (*see* Chapter 3).
   d. Gel 4—0.1 µg of protein plus one million cpm of cellular extract.
   e. Gel 5—one million cpm of cellular extract (gels 3 and 4 are the comigration).
3. After 2-D electrophoresis, soak the 2-D gels for 1 h (or overnight) in Coomassie blue fixative solution. The gels shrink in size and often turn an opaque white color.
4. Soak the gels in Coomassie blue stain solution for 15 min to rehydrate the gel, and allow for the uptake of the stain.
5. Soak the gels in destain solution for 15 min.
6. Dry the gels.

7. Mark the corners of the gel with radioactive ink (using Xs and +s works well) so the autoradiogram can be aligned with the dried stained gel. Expose the gel to XAR-5 film (Eastman Kodak, Rochester, NY) for 3 d.
8. Align the film to the gel, using the Xs and +s in the corners of the gel and film. Circle the locations of the stained spot(s). If the purified protein migrated with a radiolabeled protein, then the radiolabel should appear diffuse compared to the same spot on the gel without the purified protein (*see* **Note 3**). Sometimes a longer exposure is required to see the comigration with a minor (low-abundance) protein. Comigration alone is sufficient to identify a protein.

3.3. Identification by Enzyme Induction or Repression

Induction or repression of a protein is not sufficient to identify a protein, but can be used in combination with other identification methods to gain confidence that a certain protein spot seen on the 2-D gel is the product of a particular gene (*see* **Note 4**).

1. Grow cultures under conditions that are predicted to contain extremely different levels (or synthesis rates) of proteins of interest.
2. Radiolabel a portion of the culture and run the samples on 2-D gels (*see* Chapter 3). Comparison of the 2-D gel images should reveal which candidate proteins have altered levels in the two samples *(4)*.

3.4. Peptide Mapping

This method was first describe by Cleveland et al. *(5)*. A protein is cleaved with a specific protease, and the molecular weight of the peptides for each polypeptide represents a unique signature for the protein. This characteristic can be used to help identify a protein spot on 2-D gels (*see* **Note 5**).

1. Excise proteins out of either stained or dried 2-D gels. A razor blade or sharpened stainless-steel hypodermic needle works well.
2. Rehydrate the protein spot in soaking buffer for 30 min (change the soaking buffer once).
3. Place the swollen gel pieces in the wells of a Laemmli SDS-PAGE gel (16% T, 2.7% C separating gel with a 4.5% T, 2.7% C stacking gel).
4. Add 5 ng of protease to the each well (*see* **Note 6**).
5. Run the gel at 200 V until the dye front is about halfway through the stacking gel, and then stop electrophoresis for 30 min.
6. Resume electrophoresis at 7.5 W/gel until the dye front reaches near the bottom of the gel.
7. Stain the gel and compare the peptide patterns (*see* **Note 7**).

3.5. Selective Labeling

Selective labeling is most useful for proteins with known posttranslational modification or for proteins that uniquely bind to a certain compound (*see* **Note 8**).

Similar to the comigration techniques used for purified proteins, comigration of a radiolabeled whole-cell extract is done with the selectively labeled protein(s) to assist in precisely locating the 2-D gel position of the selectively labeled protein(s). Each extract is run alone on a 2-D gel, and the two extracts are run together on another gel.

3.6. Selective Expression from Plasmid Encoded Genes

This method works by selectively radiolabeling the proteins from cloned genes without radiolabeling proteins encoded by the host chromosome. The protein products of the cloned genes are located on 2-D gels using the comigration methods described in **Subheadings 3.2.–3.4.** Within the gene-protein database, there are many proteins that have been identified by these methods in combination with a prediction of where the gene product should migrate on the 2-D gels (*see* **Note 9**).

1. Grow the overexpression strain overnight in glucose-limiting MOPS medium containing the appropriate antibiotic to maintain the plasmid at 37°C (*see* **Note 10**). The $OD_{420 \, nm}$ of the overnight should be about 1.0.
2. Start a culture of glucose minimal MOPS medium (plus antibiotic) by diluting the overnight 10-fold.
3. Grow the culture until the $OD_{420 \, nm}$ reaches 0.8 to 1.0, and then induce the phage RNA polymerase (*see* **Note 11**).
4. After 30 min, inhibit the *E. coli* RNA polymerase by adding rifampicin (40 mg/mL stock solution made in DMSO, final concentration 0.2 mg/mL).
5. After a 30-min incubation in rifampicin, radiolabel a 1-mL portion of the culture for 10 min. The isotopes most commonly used are ^{35}S-methionine and ^{3}H amino acids (either a mixture or individual amino acids).
6. After labeling, put the culture on ice.
7. Pellet the cells by centrifugation (2–10 min in a cooled microfuge).
8. Decant the radioactive supernatant, and freeze the cell pellets at –70°C or immediately prepare for 2-D gel analysis (*see* Chapter 3).
9. Run at least three 2-D gels: one of this sample, one with a whole-cell labeling, and one with a mixture of these two samples (comigration).

4. Notes

1. If the sample is in a buffer at a concentration >0.1 mg/mL, it can be resuspended in an equal volume of lysis buffer. If the concentration of the protein in the buffer is very low, the sample can be precipitated with 80% acetone (add acetone to 80%, keep on ice for 30 min, and spin at 10,000–15,000 rpm in a microfuge), and resuspended in lysis buffer or the individual ingredients of lysis buffer can be added to the protein sample.
2. The radiolabeled protein samples used for any of the comigrations described here should have a high specific activity so that 1 µg of total protein or less is present in one million cpms. A lower specific activity could cause the protein present in

this sample to be seen on the Coomassie blue-stained gel and will make the recognition of the purified protein difficult.

3. Some proteins are present on the gel as multiple spots, usually with different migration in the first (isoelectric focusing) dimension. When identifying proteins with purified proteins as markers, it is easy to see these multiple species. All protein spots that perfectly migrate with a radiolabeled protein are identified as the product of that protein. If multiple isoelectric species are seen stained on the gel, but do not perfectly migrate with a radiolabeled protein, the extra stained spots are assumed to be the result of the purification protocol and are not considered products of the gene.

4. Using enzyme induction and repression as one criterion for identification of a protein is helpful if few proteins are affected by the induction or repression conditions. Knowing the molecular weight and p*I* of the protein and having mol-wt and p*I* markers or reference proteins on the gel can help verify the identification. In some cases, several proteins with the right response and similar molecular weight and p*I* render this method useless. A good example of why this is not sufficient information to identify a protein is shown in Fig. 2D in **ref. *4***. This figure displays the proteins induced in response to phosphorus restriction, and indicates (by letters) where some of the proteins known by genetic analysis to be induced by phosphorus restriction should migrate on 2-D gels. In many cases, there are several candidate proteins found on 2-D gels for each protein known by genetic studies.

5. In cases where the comigration test fails, but the purified protein migrated very near a protein spot from a cellular extract, a comparison of the peptide pattern from the purified protein and the protein from the cellular extract can reveal whether the two are the same protein. This situation can occur if the purified protein has become chemically modified. Another example is the situation where it is suspected that a protein is found as more than one spot, such as isoforms (*see* **ref. *6***). In the gene–protein index, there are two examples in which two different purified proteins supplied by different investigators comigrate with the same protein in the cellular extract (spot B056.5, the GroEL protein and the A-protein; F022.5, hydroxy-2-oxoglutarate aldolase, and phospho-2-keto-3-deoxygluconate aldolase) *(7,8)*. In both of these cases, peptide mapping was used to confirm that these proteins were the same.

6. Proteases of choice are those (such as V8 protease) that often yield a small number of peptides.

7. Proteins labeled with radioisotopes can also be used, but the comparison of stained and radiolabeled proteins, even of the same protein, does not usually show a complete match. More peptides will be revealed by an autoradiogram, because the sensitivity of detecting radiolabeled proteins is usually much greater, and because the radiolabeled amino acids may not be similarly represented in each peptide.

8. This method was used to aid in the identification of methylated forms of the chemotaxis proteins, Tar and Tsr, and also for penicillin binding proteins. Methods for labeling the chemotaxis proteins have been published *(9)*. Proteins modi-

fied with b_1-methylation with ^{3}H-methionine can be separated on 2-D gels. The same is true for the penicillin binding proteins *(10)*. Most methods that lead to a covalent modification with a long half-life can be identified this way.

9. Numerous methods to express proteins encoded by genes cloned into plasmids have been published. We have used minicells *(11)*, maxicells *(12)*, chloramphenicol recovery *(13)*, and expression from phage promoters using phage RNA polymerases *(14,15)*. Methods for gene expression from minicells, maxicells, and chloramphenicol have all been published in detail (*see* **refs. *11–13***) and are now seldom used because of the inherent problems of trying to overexpress genes using the cell's own transcription machinery. Expression from phage promoters using the phage RNA polymerase, like T7, T4, and SP6, is the favored method.

10. For example, we use a strain with the gene for the phage RNA polymerase integrated into the chromosome controlled by an IPTG-inducible promoter containing the expression plasmid with the cloned gene in correction orientation with the phage RNA promoter. For other stains, a cI^{ts} promoter may be used to drive the production of the RNA polymerase. The overnight cultures for these strains are grown at the permissive temperature.

11. For example, if strain BL21DE3 is the host for a plasmid with a T7 promoter, adding IPTG (stock solution 100 mg/mL, final concentration 1 mg/mL) induces the T7 RNA polymerase.

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46

Immunoaffinity Identification of 2-DE Separated Proteins

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1. Introduction

First devised in a time dominated by one-dimensional SDS-PAGE, immuno-blotting (also dubbed "Western" blotting, *[1,2]*), is now widely used in conjunction with 2-D PAGE (electrofocusing/SDS-PAGE), whose diffusion is favored by its long-awaited standardization *(3,4)* and by the coming of age of proteome science and technology *(5,6)*.

In the 2-D age, immunoblotting is still used for traditional goals, i.e., immunoaffinity identification of proteins and analysis of immune responses, and also as a genome–proteome interface technique. In fact, specific gene products can be identified in 2-D protein maps using antibodies prepared with the help of modern biotechnology on the basis of gene and cDNA sequences. When Enhanced ChemiLuminescence (ECL) detection is applied to immunoblotting, even low-abundance proteins undetectable by silver staining, such as oncogene products and cell-cycle proteins, can be monitored, e.g., **ref. 7**.

Immunoblotting results also complement with pI and M_r information to characterize posttranslational modifications of proteins. In fact, protein isoforms are identified by their crossreactivity, and then primary gene products and post-translational modifications are distinguished by comparing experimentally determined electrophoretic parameters against values predicted from amino acid sequences (e.g., **refs. 3** and **8**). Protein polymorphism owing to alternative splicing is amenable to be similarly studied, e.g., **ref. 9**.

In addition, using antibodies specific for epitopes generated by post-translational modifications, e.g., phosphotyrosine *(10,11)* and covalent adducts with

drugs *(12)*, protein targets can be identified among thousands of nonmodified proteins. The modification extent can be assessed by the number and p*I* shift of immunoreactive spots in the series, if the net charge of the protein is affected *(13)*.

2-D immunoblotting maintains the traditional steps consisting of:

1. Electrotransfer of proteins from polyacrylamide gel onto chemically resilient membrane.
2. Chemical staining of protein pattern on the *trans*-blotted membrane.
3. Saturation of the membrane.
4. Application of the primary, and then of the secondary, labeled antibody.
5. Detection of immunoreactive proteins by the label on the secondary antibody, e.g., radioactivity and enzyme activity.

In all steps, procedures developed for one-dimensional immunoblotting maintain their significance and utility. However, matching immunoreactive spots to silver-stained spots in complex, high-resolution 2-D electrophoretic patterns has become a far more difficult task than matching bands in one-dimensional separations. As a consequence, chemical staining of total protein pattern on the *trans*-blotted nitrocellulose or on equivalent membrane plays a crucial role in 2-D immunoaffinity identification. Matching itself is better carried out on digitized images with adequate computer and software than by physically superimposing the chemilumiscent film or the membrane to the silver-stained gel (cf. **Subheadings 3.4.** and **3.5.**).

The cost of reagents per sample is also higher than with the one-dimensional procedure, since all volumes and surfaces are larger. As a preliminary step, it is convenient to find out antibody working dilution by one-dimensional immunoblotting. In the same way one can assess the need for a blank incubation with secondary antibody, omitting the primary antibody. Costs for 2-D gel run and electrotransfer reagents can also be reduced by reusing the electroblotted membrane consecutively with different antibodies (cf. **Subheading 3.4.**, stripping). All reagents can be saved if "multiple immuno-2-D blotting," i.e., simultaneous application of several primary antibodies, can be performed. In this procedure, unambiguous immunodetection is allowed by different 2-D electrophoretic parameters of proteins *(7)*.

2. Materials
2.1. Equipment

1. Blotting apparatus: transfer cell, gel holder, magnetic stirrer, refrigerated thermostatic circulator unit.
2. Power supply.
3. Rocking agitator.
4. Computing densitometer.
5. Workstation with a computer program for 2-D gel analysis.

2.2. Reagents

1. Distilled water.
2. Nitrocellulose membrane.
3. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol. Do not adjust pH; it is about 8.3.
4. Filter paper for blotting (Whatman 17 Chr).
5. Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid (TCA).
6. Phosphate-buffered saline (PBS): 0.15 *M* NaCl, 10 m*M* NaH_2PO_4, bring to pH 7.4 with NaOH.
7. Blocking solution: 3% (w/v) non-fat dry milk in PBS, Triton X-100 0.1% (w/v).
8. Primary antibody solution (primary antibody, opportunely diluted in blocking solution).
9. Second antibody solution (secondary antibody, opportunely diluted in blocking solution).
10. Washing solution: Triton X-100 0.5% (w/v) in PBS.
11. 0.05 *M* Tris-HCl, pH 6.8.
12. Amersham ECL kit, cat. no. RPN 2106 (Amersham International plc, Little Chalfont, UK).
13. Saran Wrap or other cling films.
14. X-ray films, 18 × 24 cm (Amersham Hyper film ECL, cat. no. RPN 3103).
15. Developer and fixer for X-ray film (developer replenisher; fixer and replenisher, 3 *M*, cat. no. XAF 3 and XAD 3) (3*M* Italia S.p.A., Segrate, Italy).
16. Stripping buffer: 100 m*M* 2-mercaptoethanol, 2% (w/v) SDS, 62.5 m*M* Tris-HCl, pH 6.7.

3. Methods
3.1. Transfer

To avoid membrane contamination, wear gloves during all the steps of the experiment.

1. Prepare the transfer buffer, and cool it to 4°C before the end of the electrophoretic run (*see* **Notes 1–3**).
2. Cut to the dimensions of the gel, two pieces of filter paper and one piece of nitrocellulose/gel (*see* **Note 4**).
3. Following electrophoresis, wash the gel in distilled water and then equilibrate it in transfer buffer. 10 to 15 min are the ideal time for 1.5-mm gels (*see* **Note 5**).
4. Soak the nitrocellulose membrane for 15–20 min in transfer buffer. Also wet two "Scotch-Brite" pads/gel and filter papers in transfer buffer.
5. Assemble the "sandwich" for transfer in this order: fiber pad, filter paper, nitrocellulose, gel, filter paper, fiber pad. Remove all air bubbles between membrane and gel and between paper and gel.
6. Put the blot sandwich in the gel holder, and hold it firmly to ensure a tight contact between gel and membrane (*see* **Note 6**).

7. Fill the cell with transfer buffer, and place a stir bar inside the transfer cell so that the buffer is stirred during electrotransfer (*see* **Note 7**).
8. Place the gel holder in the transfer cell with the sandwich oriented as follows: ANODE/fiber pad, filter paper, nitrocellulose, gel, filter paper, fiber pad/CATHODE.
9. Carry out blotting at 100 V with a constant voltage for 1 h (*see* **Note 8**), refrigerating the buffer to 4°C (*see* **Note 9**).
10. After electrotransfer, disassemble the blotting apparatus and remove the nitrocellulose membrane. To mark the orientation of the membrane, cut away the lower right corner, corresponding to low M_r, high pH.

The membrane can be processed immediately for immunoblotting or can be air-dried and stored at –20°C, within parafilm sheets for extended periods *(14)*.

3.2. Staining of Total Protein Pattern on Membrane

1. Before the immunodetection, stain the nitrocellulose membrane in 0.2% (w/v) Ponceau S in 3% w/v trichloroacetic acid for 3 min *(15)* (*see* **Note 10**).
2. Destain with several changes of distilled water to diminish background color. Since the red spots will disappeared in the blocking step, circle with a waterproof pen some spots that will be used as landmarks to match total protein pattern on nitrocellulose vs immunoreactive pattern and vs silver stained polyacrylamide gel pattern (*see* **Note 10**).

3.3. Immunodetection

3.3.1. Incubation with Antibodies

All steps are carried out at room temperature and with gentle agitation on a rocking agitator.

1. Block nonspecific binding sites in the membrane with three washing steps, each for 10 min, in blocking solution (*see* **Notes 11** and **12**).
2. Incubate overnight in the primary antibody solution at the suitable dilution (*see* **Note 13**) in blocking solution.
3. Wash for 3 × 10 min in blocking solution.
4. Incubate for 2 h in the secondary antibody solution.
5. Wash for 3 × 10 min in blocking solution.
6. Wash for 30 min in washing solution.
7. Wash 2 × 30 min in 0.05 *M* Tris-HCl, pH 6.8.

After this step, one can go forward with ECL detection. Alternatively, one can choose detection with the chromogenic substrate (*see* **Note 14**).

3.3.2. Enhanced Chemiluminescent Detection

To detect the immunoreactive spot(s) with Amersham ECL kit, it is necessary to work in a darkroom and to wear gloves to prevent hand contact with film.

1. Mix equal volumes of detection reagent 1 and detection reagent 2 from the Amersham ECL kit, and immerse the membrane in this solution for 1 min, ensuring that all the surface of the membrane is covered with solution (*see* **Note 15**).
2. Place the membrane on a glass, and cover it with a layer of Saran Wrap.
3. Cut away a corner from a piece of autoradiography film to define its orientation (*see* **Subheading 3.1.**, **step 10**). Superimpose the autoradiography film on the nitrocellulose membrane beginning from the upper left corners. Nitrocellulose membrane and X-ray film may have different dimensions. Superimposing at the upper left corner for ECL impression will allow subsequent matching of images (*see* **Subheading 3.5.**, **step 2**).
4. Expose the film for a time variable from 5 s to several minutes. It is convenient to begin with short exposure, develop the film, and then try longer exposures, if necessary.
5. Develop the film with the suitable reagents (*see* **Note 14**).

3.4. Stripping

At the end of a cycle of immunodetection, it is possible to strip the membrane with indicated solution and to carry out subsequent cycles incubating with different primary antibodies (*see* **Note 16**). The procedure for the stripping we use is:

1. Incubate the membrane in stripping buffer at 70°C for 30 min, with occasional shaking.
2. Wash the nitrocellulose for 2 × 10 min in large volumes of washing solution at room temperature.
3. Block the membrane and perform immunodetection as described in **Subheading 3.3.**

3.5. Matching

For an accurate matching process, we use a computer program (*see* **Note 17**) that permits matching the digitized images, using as landmarks the spots stained with Ponceau S (*see* **Note 18**).

To perform this operation, we suggest the following procedure (*see* **Fig. 1**):

1. Scan the ECL-developed film, the Ponceau S-stained nitrocellulose membrane, and the silver-stained gel of the same sample with a computing densitometer with a sufficient resolution (*see* **Notes 19** and **20**).
2. Rotate left–right the nitrocellulose membrane, with an appropriate program, in order to have the three images with the cut lower corner on the right. In fact, the nitrocellulose membrane has the spots only in one face, and the scanning process generates an image with the cut lower corner placed on the left.
3. Stack together the film and nitrocellulose membrane images, aligning the upper left corners and the two corresponding borders, and placing the cut lower right corner in the same orientation for both.
4. Add "manually," with appropriate software tool, the Ponceau S spots chosen as landmarks onto the image of the ECL film.
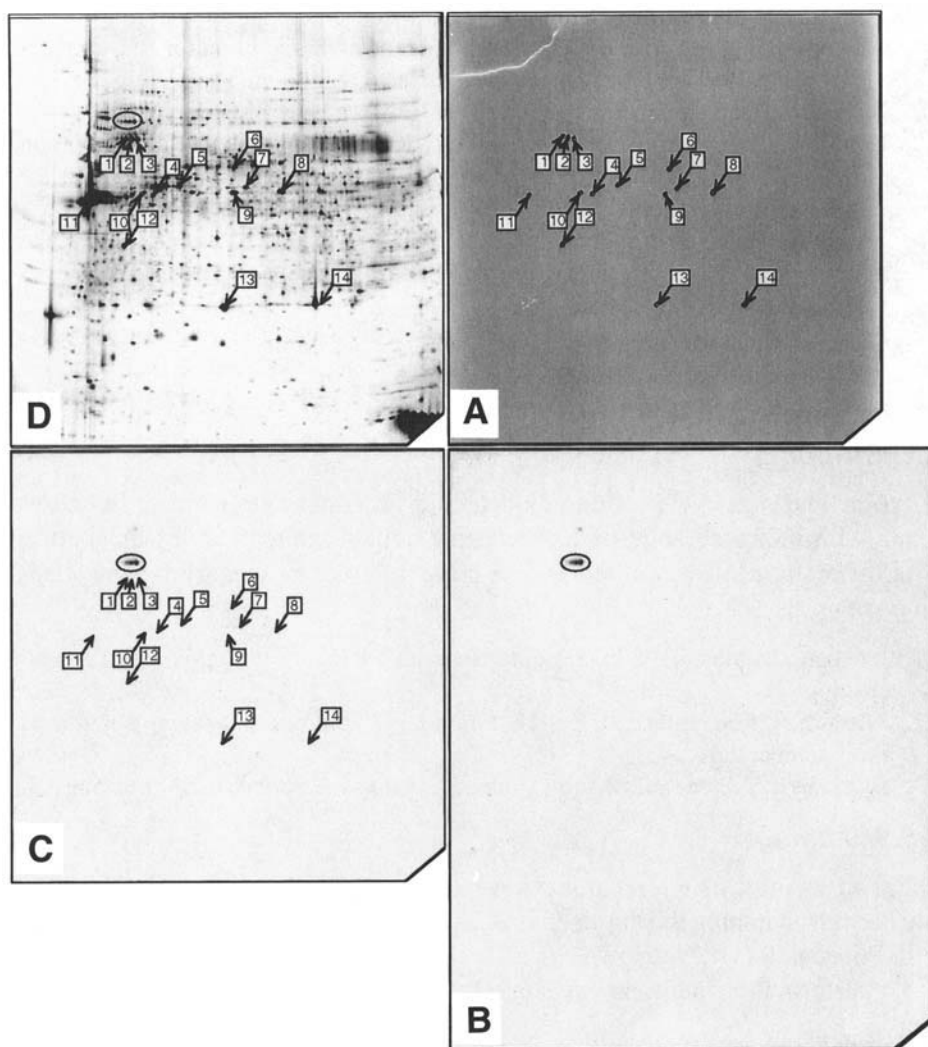
Fig. 1. Immunoaffinity identification of S1 ribosomal protein in the *Chlamydia trachomatis* serovar L2 protein map, comprised within pI 3.5–9, M_r 10- to 200-kDa window. Electrophoretic conditions as in **ref. *8***. Immunoblotting and ECL detection were reported (*see* **Subheading 3.**). (*Chlamydia* Elementary Bodies were provided by G. Ratti, Chiron-Biocine Research Centre, Siena, Italy; monoclonal antibody [MAb] to S1 ribosomal protein was provided by S. Birkelund, Department of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark). **(A)** Digitized image of Ponceau S-stained nitrocellulose membrane. Arrows and number indicate spots chosen as landmarks, being recognizable both in the *trans*-blotted nitrocellulose and in the silver-stained gels. **(B)** Digitized image of impressed ECL film. Immunoreactive isoelectric series is circled. **(C)** Image constructed in order to adding landmarks in

5. Find the spots on the gel corresponding to landmarks on the film, and modify the size of the silver nitrate image adjusting it to the smaller one of film by the mean of adequate software. Actually the gel is larger than the film owing to silver-staining procedure.

6. Stack together the equalized ECL film and gel images, superimpose the landmarks carefully, and run the automatic match program. This operation permits automatically highlighting the silver-stained spots paired with the immunoreactive ones present on the ECL film.

4. Notes

1. We perform electrotransfer from gels to nitrocellulose membrane, following a "wet" method. In a tank or wet apparatus, the gel is submerged in a large volume of buffer during the transfer. We use Bio-Rad transfer cell with 3 L of transfer buffer. "Semidry" electroblotters require smaller volumes of buffer, since membrane and filter paper only are to be wet. The semidry procedure is faster. However, the wet method is recommended when antigen is present in small quantities (as low-abundance spots in 2-D gels) and/or its molecular weight is high *(16)*.

2. The transfer buffer we use was first described by Towbin et al. *(1)*. Methanol is toxic, and it can be omitted *(17–19)*. Still, we use it to reduce swelling of the gel during transfer and to increase the binding of proteins to nitrocellulose *(2,17,20)*. Some recipes recommend the addition of low concentration of SDS to the buffer to help the transfer of high-mol-wt proteins *(16)* and to improve the transfer of a variety of proteins *(17)*. However, SDS reduces the amount of protein bound to the membrane *(2)* and may adversely affect immunoreactivity by inhibiting renaturation of antigenic sites *(21)*.

3. Reagent-grade methanol only is to be used because trace impurities in methanol can increase the conductivity of transfer buffer and decrease transfer efficiency.

4. Polyvinylidene difluoride (PVDF) may also be used *(22,23)*. Remember that unlike nitrocellulose, PVDF is a hydrophobic membrane, and it must be prewetted in methanol before use with aqueous solution. The buffer generally used to transfer proteins to PVDF is 10 m*M* 3-[cyclohexylamino]-1-propanesulfonic acid, 10% (v/v) methanol, pH 11.0 *(24)*.

5. Electrotransfer is usually carried out immediately after the electrophoretic run from unstained gels. However, transfer of proteins from polyacrylamide gels after Coomassie blue or silver staining has also been reported *(25,26)*. Proteins can also be transferred for immunodetection from gels previously stained in a reverse (negative) way, e.g., with imidazole-zinc salts *(27)*.

In these procedures, immunoreactivity pattern on the membrane and total protein pattern can be obtained from the same gel from which spots have been *trans-*

A onto B, the process of image-stacking being at the upper left corner. Exceeding surface in the ECL films is cut by computer. **(D)** Digitized image of silver-stained gels. Size is equalized to that of image C. Recognition of landmarks allows recognition of immunoreactive spots.

blotted, facilitating matching even with not easily reproducible 2-D separations. These procedures have become less interesting, since the introduction of highly reproducible 2-D separation protocols *(3,4)* and of computer-aided matching (cf. **Subheading 3.5.**).

6. It is important that the layers of sandwich are firmly held together to have a good transfer without distortion of the proteins spots.

7. The stirring of the buffer ensures uniform temperature and conductivity during electrotransfer.

8. The transfer efficiency is adversely affected by high-mol-wt and basic p*I* of proteins. Therefore, while attempting to transfer slow proteins, it may happen that fast proteins cross the nitrocellulose membrane and are lost. In these cases, one can use two stacked membranes or membranes with smaller pore diameter, which would also prevent loss of small polypeptides during membrane manipulation *(28,29)*. *See* **ref. *30*** for information on blotting on various membranes.

   Some low-mol-wt, basic proteins, such as histones, lysozymes, cytochromes, and so on, do not transfer well because they may be near their pI in currently used buffers, since SDS is lost during the transfer in methanol. Transfer of these proteins can be improved, without impairing transfer of other proteins, by introducing a more basic transfer buffer and/or omitting the equilibration (**Subheading 3.1.**, **step 3**) *(31)*. Alternative buffers have also been proposed (e.g., **ref. *32***).

9. When the transfer is conducted at high voltage, it is necessary to refrigerate the transfer tank with a thermostatic circulator. If possible, avoid transfer in the cold room.

10. Chemical staining of proteins patterns *trans*-blotted onto the nitrocellulose or other membrane plays an important role in 2-D immunoaffinity identification, since it provides "landmark" spots to match immunoreactivity patterns to silver-staining patterns (cf. **Subheading 3.5.** and **Note 18**). Several staining procedures can be chosen. This step is usually carried out before the incubation of transblotted membranes with antibodies, employing dyes (e.g., Ponceau S, fast green, amido black) or metal chelates, which do not interfere with protein immunoreactivity *(28,33–36)*.

    Other systems (e.g., with colloidal gold *[37,38]*) are applied after immunodetection. The latter approach is possible if membrane saturation is achieved by Tween-20, omitting proteins *(39)*. Staining with india ink can also be used after immunodetection *(40)*.

    Staining with substances, such as Ponceau S, fast green, and metal chelates, is reversible, eliminating interference in the immunoreactivity pattern obtained with chromogenic substrates. Permanent staining, e.g., with amido black, can be used if immunoreactivity pattern is collected from ECL-impressed films.

    When radioactive labeling is possible, most accurate total protein patterns can be collected from *trans*-blotted membranes by phosphor-imaging (e.g., **ref. *11***).

11. For a 16 × 18-cm membrane, we use 50 mL of solution in each washing and incubation step. Volumes can be proportionally adjusted to other membrane dimension. It is important that the membrane is entirely soaked in solution during the washing and incubation step.

We perform all steps of immunodetection in a flat glass vessel. Rotating glass cylinders are also convenient. Do not incubate membrane in sealed plastic envelopes, because it results in a very high background in ECL.

12. Our blocking procedure is suitable for routine use. However, special conditions and reagents are required for immunoblotting with some antibodies, for example, antiphosphotyrosine antibodies *(11,41)*. Information on different blocking conditions can be found in refs. *(42–45)*. Blocking with a nonionic detergent, such as Tween-20, without added protein has also been introduced with the advantage that after immunodetection, the blot can be stained for total protein pattern *(39,46)* (*see* **Note 10**). On the other hand, it has been found that blocking with detergent alone may cause loss of transblotted proteins *(47,48)*.

13. Optimal dilution of the primary and secondary antibody should be determined by immunoblotting of mono-dimensional gels, or dot-blot analysis can be used. Working solutions of antibodies can be stored at –20°C and used several times *(49)*.

14. In case chemiluminescence is too strong or background is too high, one can switch to detection with a chromogenic substrate. We use 4-Chloro1-naphthol *(50)* as chromogenic substrate, according to the following protocol:
    a. After ECL detection (or after **step 7** of **Subheading 3.3.1.**), wash the membrane briefly with Tris-HCl, 0.05 *M*, pH 6.8.
    b. Soak the membrane in developing solution (20 mL Tris-HCl 0.05 *M*, pH 6.8) 7 μL H_2O_2 30% (v/v); 5 mL 4-chloro 1-naphthol 0.3% in methanol until the color appears.
    c. Stop the reaction with washes in distilled water.
    d. Air-dry the membrane and photograph it as soon as possible, because the color fades with time.

15. For a 16 × 18-cm membrane, use 7.5 mL of reagent 1 and 7.5 mL of reagent 2.

16. Stripping of antibodies also elutes antigens from the membrane, and signal intensity decreases in successive cycles. As alternatives to stripping, one can use:
    a. Different chromogenic substrates for peroxidase at each cycle (rainbow blotting *[49]*).
    b. ECL and inactivating peroxidase after each cycle *(49)*.
    c. Different labels and detection methods at each cycle *(51)*.

17. To perform the matching process, we use the software Melanie II release 1.2 from Bio-Rad.

18. Matching can also be done by simple eye inspection directly on nitrocellulose and ECL film when the sample contains relatively few spots, all of them detectable by chemical staining of nitrocellulose. In the majority of cases, samples are very complex, and many low-abundance proteins occur. In these cases, matching by computer is mandatory in order to identify immunoreactive spots in silver-stained patterns. The following manual procedure is suggested:
    a. Match the exposed film with nitrocellulose membrane, aligning the upper left corner and the two corresponding borders and placing the cut lower right corner in the same orientation for both.

b. Using a waterproof pen, mark the other two borders of the nitrocellulose on the film, and transfer the chemically stained spot present on nitrocellulose on the ECL film in order to use them as landmarks for the next matching with the silver nitrate-stained gel.

c. Nitrocellulose membrane and film maintain the initial size, but the size of the gel increases after silver staining. Size equalization can be obtained by photographic or photocopy procedures.

d. On a transilluminator, match all the landmarks with the corresponding spots on the silver nitrate-stained gel to identify the immunoreactive spots.

19. We use a computing densitometer 300 S from Molecular Dynamics with a resolution of 4000 × 5000 pixels, 12 bits/pixel, which generate 40-megabytes images on 16 bits.

20. The silver-stained image used for matching can be taken from your archive of files or from images available in Internet, provided that the identical electrophoretic procedures have been applied. The possibility of matching images deriving from different 2-D electrophoretic procedures has been studied by Lemkin *(52)*.

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47

2-DE Spot Amino Acid Analysis
with 9-Fluorenylmethyl Chloroformate

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1. Introduction

Amino acid analysis is a powerful and sensitive technique for the determination of the amino acid composition of proteins. In the past, the automation of amino acid analysis was based on postcolumn ninhydrin analysis, e.g., the Beckman 6300. However, a demand for high sensitivity and high sample throughput has led to the development of precolumn derivatization of amino acids and subsequent separation by reverse phase-high performance liquid chromatography RP-(HPLC), e.g., GBC AMINOMATE system. Although the chromophoric or fluorogenic derivatives allow greater sensitivity (100 fmol to low pmol range of protein), protein hydrolysis remains the limiting factor to high sensitive amino acid analysis. At the 5- to 20-pmol level, various contaminants influence the accuracy of both the qualitative and quantitative aspects of the analysis. Therefore, correct procedure and careful manipulation are required to achieve a successful analysis.

The method described in this chapter is based on the 9-fluorenylmethyl chloroformate (Fmoc-Cl) derivatization of amino acids, produced by acid hydrolysis of membrane (such as polyvinylidene difluoride [PVDF], Teflon, hyperbond) blotted proteins separated from one-dimensional (1-D) or two dimensional (2-D) gel electrophoresis. Fmoc-Cl readily reacts with the free α amino group of both primary and secondary amino acids to form highly fluorescent fluorenylmethyloxycarbonyl amino acids (Fmoc) derivatives, which are separated by reverse-phase HPLC. The reaction (shown in **Fig. 1**) occurs rapidly at alkaline pH and ambient temperature, and the products are stable over many hours and can be detected at the 500 fmol level.
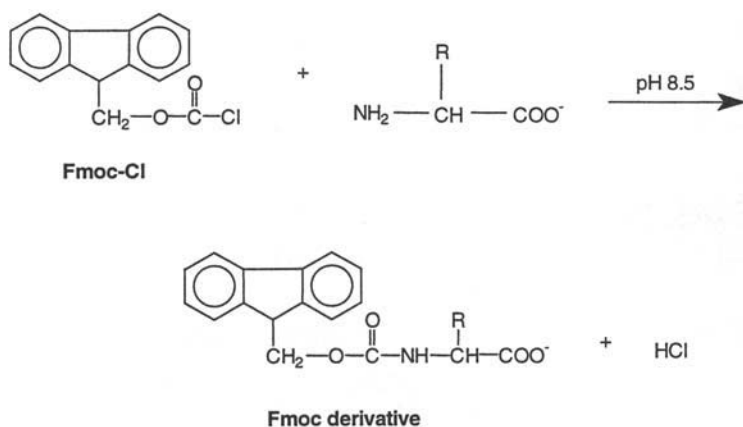
Fig. 1. Reaction of Fmoc-Cl with α amino group of amino acids to form highly fluorescent derivatives.

Fmoc-Cl was first used as a precolumn derivatizing agent for amino acid analysis by Einarsson et al. *(1)*, and the procedure was simplified by Haynes et al. *(2,3)* with the Fmoc derivatives being separated by reverse-phase HPLC. The simplified method avoided removal of the Fmoc reagent, since it was sequestered by the addition of an excess alkaline hydroxylamine. The Fmoc hydroxylamine product remained soluble in the reaction mixture and did not interfere with the chromatography of the amino acid derivatives. The treatment with alkaline hydroxylamine also served to convert the disubstituted derivatives of histidine and tyrosine into the stable monosubstituted derivatives. More recently, improvements in chromatography and automation of the process have resulted in further increases in sensitivity and sample throughput *(4)*, which make feasible large-scale proteome studies with the amino acid composition being used as the primary means of protein identification through database searching *(5,6)*.

2. Materials

The method described here was carried out using GBC (Australia) Automated AMINOMATE system, but equivalent instrumentation can be used. All the chemicals and apparatus are essentially those described by Tarr et al. *(7)* and Meyer et al. *(8)*. For the use of GBC automated Aminomate system, all materials are also described in detail by Ou et al. *(4)* and Yan et al. *(5)*.

2.1. Hydrolysis Reagents

1. Hydrochloric acid: AR-grade, BDH, cat. no. 45002, or constant boiling temperature 6 *N* HCl, Pierce, cat. no. 24309.
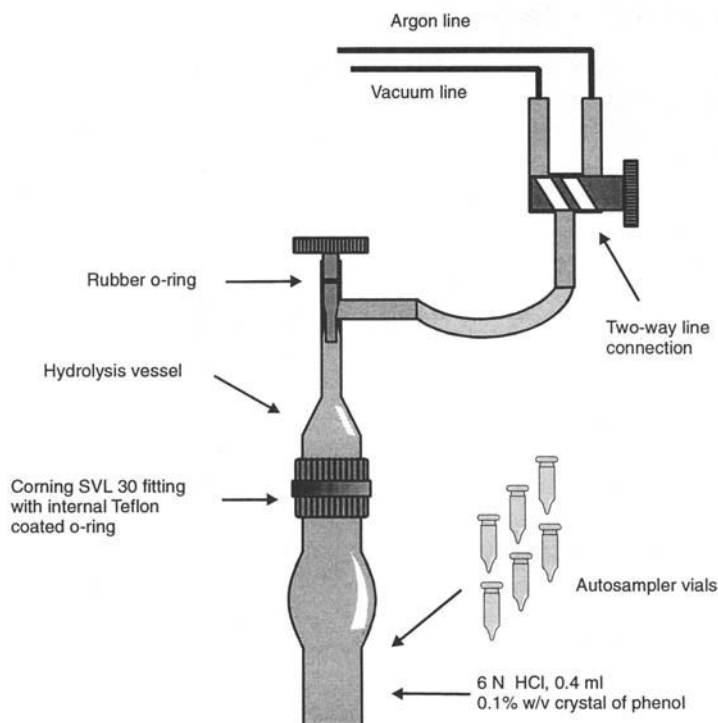
Fig. 2. The design of the hydrolysis vessel. The vessel incorporates a belly-bottomed flask threaded at the top to match a Corning SVL30 fitting. The SVL30 fitting has a permanently attached vacuum tap with a Teflon piston that incorporates an rubber o-ring. A Teflon-coated o-ring is used to seal the belly bottom to the top, inside SVL30 fitting. Hydrolysis vessels can be obtained from the authors (http://www.bio. mq.edu.au/APAF).

2. Phenol: ultra-pure, ICN, cat. no. 800672, stored at 4°C.
3. Trifluoroacetic acid (TFA): protein sequencer grade, ICN, cat. no. 190281.
4. Argon gas: ultra-high purity.
5. Low temperature (–80°C or –110°C) Savant vacuum trap (or equivalent) with a vacuum gage: essential for setting up the hydrolysis as well as drying samples after hydrolysis.
6. Two-way line connection: *see* **Fig. 2**.
7. Hydrolysis vessel: *see* **Fig. 2**. It can be obtained from the authors by contacting us at http://www.bio.mq.edu.au/APAF.
8. Oven: set at 160°C in a well-vented area.
9. Fume hood: should be dedicated to the use of amino acid analysis.
10. Eppendorf crystal tips for 10 μL:these are the only tips that can reach to the bottom of the autosampler vials, Crown Scientific, cat. no. 4810-0.5.

2.2. Derivatization Reagents

1. Acetonitrile: HPLC-grade, Mallinckrodt, cat. no. 2856.
2. Acetic acid glacial: AR-grade, Mallinckrodt, cat. no. 8817KXCM.
3. Fluorenylmethyl chloroformate: Sigma, cat. no. F0378, moisture-sensitive, stored dry at 4°C.
4. Hydroxylamine hydrochloride (NH_2OH-HCl): Sigma, cat. no. H2391, air- and moisture-sensitive, kept at room temperature.
5. 2-(Methylthio)-ethanol: Sigma, cat. no. M9268, harmful liquid, open in fume hood.
6. Sodium hydroxide (NaOH): AR-grade, ICN, cat. no. 153495, kept in plastic bottle.
7. Boric acid: AR-grade, ICN, cat. no. 195074.
8. Fresh Milli-Q or equivalent water: Millipore.
9. Vial crimper: necessary to crimp the caps correctly onto vials.
10. Autosampler consumables: 2-mL glass, with flat-bottom, wide-neck vials, used with crimp caps, cat. no. 2-DV, Chromacol; aluminium crimp cap fitted with a white silicone/red PTFE seal, cat. no. 11-AC-ST15, Chromacol; 200-µL glass vials, 5 × 32 mm, tapered low-volume insert for use with 2-DV, made from inert Chromacol Gold™ grade glass, cat. no. 02-MTVWG, Chromacol; polyethylene self-centering support device for use with 02-MTVWG, cat. no. MTS-1, Chromacol. **Note:** 2-DV vials and MTS-1 supports are re-usable.
11. Autosampler spares: injection needle, cat. no. 62-0390-00; needle wash assessory, cat. no. 240-0275; syringe (100 µL), cat. no. 240-011200; SHP seal, cat. no. 100-336100; rotor seal, cat. no. 100-337500. All are from GBC Scientific Equipment, Dandenong, Victoria, Australia.

2.3. Chromatography Reagents

1. Anhydrous ammonium monohydrogen phosphate ($[NH_4]_2HPO_4$): ICN, cat. no. 152498.
2. Anhydrous dihydrogen phosphate ($NH_4H_2PO_4$): ICN, cat. no. 150110.
3. Methanol: HPLC-grade, Mallinckrodt, cat. no. 3016.
4. Amino acid standards H: protein hydrolysate standard in 0.1 *N* HCl, containing a solution of 17 amino acids (2.5 m*M* each, except cystine, which is 1.25 m*M*), Sigma, cat. no. A9781.
5. L-Hydroxyproline: used for internal standard, Sigma, H1637.
6. Hypersil C18 reverse-phase column: 150 × 4.6 mm ID 5 µm, Keystone Scientific, Bellefonte, PA.
7. PVDF filter: 0.45 µm, Millipore.
8. In-line filter (2 µm): Upchurch, cat. no. 100-10.
9. GBC Automated AMINOMATE system LC-1150 pump; LC-1250 fluorescence detector (excitation $\lambda = 270$ nm, detection $\lambda = 316$ nm); LC-1650 advanced autosampler; Gastorr GT-104 degasser; the system is controlled by GBC WinChrom windows software available from the GBC Scientific Equipment, Dandenong, Victoria, Australia.
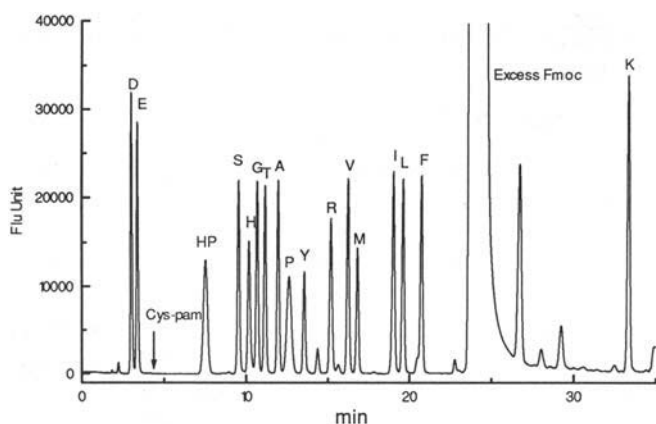
Fig. 3. A typical separation of standard amino acids.

3. Methods

3.1. Acid Hydrolysis

Protein hydrolysis utilizes heating with one or more acids to hydrolyze proteins to amino acids. Not all amino acids are routinely recovered from this procedure. Asparagine and glutamine are deamidated quantitatively to aspartic and glutamic acid. No attempt is normally made to quantitate these separately. Tryptophan, cysteine, and cystine are largely destroyed during a normal 6 *N* HCl hydrolysis. Analyses for tryptophan may be carried out using modified conditions with a few percent β-mercaptoacetic acid in the 6 *N* HCl *(9)* or employing sodium hydroxide with hydrolyzed starch added as a scavenging agent *(10)*. Cysteine content can be determined using alkylation of the sulfhydryl group to form a more stable thioether prior to acid hydrolysis *(11,12)*. The hydrolysis procedure described here recovers 17 amino acids (asparagine and glutamine are deamidated to their corresponding acids, tryptophan is not included, and cysteine is modified to cysteine-S-propionamide by alkylation with acrylamide, and converted into its acid form, cysteine-S-propionic acid, after acid hydrolysis). The elution positions are indicated in **Fig. 3**.

Protein hydrolysis is traditionally viewed as a difficult technique and is often thought to be the major source of error in amino acid analysis. However, with practice and suitable internal controls, the technique is very simple and reliable, and minor variations in hydrolysis efficiency can be controlled. The design and reliability of the hydrolysis vessel are particularly important. The vessel has to withstand high temperature in the presence of strong acid vapor, and hold positive and negative gas pressure during each hydrolysis run. In the absence of affordable commercially available hydrolysis stations, a specially constructed hydrolysis vessel was developed for hydrolysis of small amounts

of proteins (**Fig. 2**). This vessel design is essentially that of Meyer et al. *(8)* and can be made by any glassblower.

3.1.1. Hydrolysis Procedure

1. Excise protein spot from membranes. Place into autosampler vial and include known proteins as controls that are from either the same PVDF blot or a different blot (*see* **Note 1**).
2. Add 400 μL of 6 *N* HCl, and a crystal of phenol (about 0.1%, w/v) into the bottom of the hydrolysis vessel (*see* **Note 2**).
3. Place up to 18 autosampler vials carrying protein spots into the vessel. If fewer than 12 samples are to be hydrolyzed, blank vials should be used for support. Make sure the vials of top layer are sitting between the vials of bottom layer and **not** directly on the vials. Otherwise, vials will be covered, and acid vapor cannot contact the PVDF spot.
4. Assemble the vessel tightly. Connect vessel to the two-way argon and vacuum line.
5. Evacuate (vacuum) for 10 s (the acid will boil during this procedure; the vessel is evacuated to 3 mtorr), and flush with argon for 10 s to remove oxygen (*see* **Note 3**).
6. Repeat vacuum and argon flush (10 s each) twice, and finally evacuate for 10 s and seal the vessel (*see* **Note 3**).
7. Place evacuated vessel into the 160°C oven for 1 h. We use a preheated aluminum holder so that the oven temperature remains constant (*see* **Note 4**).
8. After heating, remove the hydrolysis vessel from the oven, and open the vacuum tap immediately within a fume hood (*see* **Notes 5** and **6**).
9. Remove vials from the vessel into Eppendrof tubes (1.5 mL) with lids open, and place into vacuum centrifuge for 10 min to evaporate residual acid (*see* **Note 7**).

3.1.2. Extraction of Amino Acids from PVDF

1. Prepare the extraction solution as described in **Table 1**. Add 170 μL of the solution into each vial containing a PVDF spot.
2. Sonicate for 10 min. Eppendorf tubes are placed upright in ultrasonic bath using a foam floater with lids closed, so that vials inside tubes are free from contamination.
3. Remove the PVDF spot from each vial with a fresh stainless-steel hypodermic needle (*see* **Note 8**).
4. Lyophilize the solution in vacuum centrifuge. The dried hydrolysate is ready for derivatization.

3.2. Chromatography

3.2.1. Derivatization (*see* **Note 9**)

1. The preparation of borate buffer, derivatizing reagents and internal needle wash are described in **Table 1** (*see* **Notes 10–12**).
2. Dissolve the hydrolysate in 10 μL of 250 m*M* borate buffer, pH 8.8, using an Eppendorf crystal tip, which allows good access to the narrow bottom of the autosampler vial.
3. If samples are being derivatized automatically, place the vial onto MTS-1 support within 2-DV glass vial. Crimp the vial with an aluminum cap fitted with a

Table 1
Preparation for Extraction Solution, Borate Buffer,
Derivatization Reagents, and Internal Needle Wash

Solution	Preparation	Storage and note
Extraction solution	Make 0.1% TFA: 1 µL TFA + 999 µL H_2O Mix acetonitrile, H_2O and 0.1% TFA in the proportion of 10:5:2., i.e., acetonitrile 100 µL, H_2O 50 µL, and 0.1% TFA 20 µL	Make fresh before use Add 170 µL for each vial carrying protein PVDF spot
250 mM borate buffer, pH 6.5	Add 80 mL H_2O to 1.55 g boric acid Adjust pH to 8.5 with 1 M NaOH solution Make up to 100 mL with H_2O	Kept for 1 mo at 4°C Aliquot into small volumes to avoid crosscontamination
Fmoc reagent (4 mg/mL)	Add 2.5 mL acetonitrile to 10 mg Fmoc	Stored at 4°C for 1 week
Cleavage reagent	Stock solution: 0.85 M NaOH: Add 10 mL H_2O to 0.34 g NaOH 0.5 M NH_2OH-HCl: Add 10 mL H_2O to 0.35 g NH_2OH-HCl Working solution: total 1 mL Mix 680 µL 0.85 M NaOH, 150 µL 0.5 M NH_2OH-HCl and 20 µL 2-(methylthio)-ethanol	Stock NH_2OH-HCl solution can be kept for 1 mo at 4°C Working cleavage reagent must be made fresh prior to each use NaOH solution should be stored in plastic bottle 2-(Methylthio)-ethanol is very dense, mix the solution thoroughly
Quenching reagent	Mix 2 mL acetic acid and 8 mL acetonitrile	Can be stored for up to 6 mo at 4°C
Internal needle wash	Mix 250 mL acetonitrile and 750 mL H_2O	Kept at room temperature for up to 6 mo

white silicone/red PTFE seal, and immediately load into the autosampler tray for derivatization. The derivatizing procedures (pretreatment file) are listed in **Table 2**.

4. If samples are being derivatized manually, the procedures are as follows.
5. Dissolve protein hydrolysate in 10 µL borate buffer, pH 8.8, and then add the following:
   a. 10 µL Fmoc reagent, wait for 1 min (mix).
   b. 10 µL Cleavage reagent, wait for 4 min (mix).
   c. 10 µL Quenching reagent (mix).
6. After the completion of manual derivatization, do **step 3** to seal the vials. The derivatized solution is ready for HPLC analysis.

Table 2
Multitasking Program (Pretreatment File)
for Automated Derivatization and Injection

	Command	Vol., μL	Vial position	Parameter
1	External Wash—Duration			5 s
2	Vial—Get	10 (needle wash)	1	
3	Needle—Dump Content			
4	Vial—Get	10 (needle wash)	1	
5	Vial—Put	10 (needle wash)	2 (waste vial)	
6	External Wash—Now			
7	Vial—Get	2 (air)		
8	Vial—Get	30 (needle wash)	1	
9	Vial—Get	2 (air)		
10	Vial—Get	10 (Fmoc reagent)	4	
11	Vial—Put	12 (Fmoc + air)	[a]	
12	Vial—Get	20 (air)		
13	Vial—Put	20 (air)	[a]	
14	Vial—Put	10 (needle washing)	2	
15	External Wash—Now			
16	Operation—Wait			60 s
17	Vial—Get	2 (air)		
18	Vial—Get	10 (cleavage reagent)	5	
19	Vial—Put	12 (cleavage + air)	[a]	
20	Vial—Get	20 (air)		
21	Vial—Put	20 (air)	[a]	
22	Vial—Put	10 (needle wash)	2	
23	External Wash—Now			
24	Operation—Wait			200 s
25	Vial—Get	2 (air)		
26	Vial—Get	10 (quenching reagent)	6	
27	Vial—Put	12 (quenching + air)	[a]	3
28	Vial—Get	20 (air)		
29	Vial—Put	20 (air)	[a]	
30	Vial—Put	10 (needle wash)	2	
31	External Wash—Now			
32	Operation—Park Inject	[b]	[a]	

[a]Represents the vial position of the sample to be automated, derivatized, and injected.
[b]Represents the injection volume between 5 and 40 μL.

3.2.2. Multitasking of Derivatization and Chromatography

During normal operation of the GBC AMINOMATE system, the sequence of events is first to equilibrate the column, then derivatize the sample, and

finally inject the sample and run the separation gradient. This sequence of events takes a total of 50 min/sample. In order to increase the throughput of the system, a multitasking method has been devised to run a separation gradient of one sample and simultaneously derivatize the next sample to be analyzed *(4)*. This procedure reduces the per-sample run time to 38 min, giving a time saving of 12 min/sample (24%), and also reduces the total solvent consumption per run by 24% from 50 to 38 mL.

The multitasking program (pretreatment) is shown in **Table 2**. With the newly released autosampler (LC1650) a new "pretreatment editor setting dialog box" was created in the new version (1.2) of WinChrom software. This dialog box enables the selection of when to start processing the next pretreatment file in the batch table during the current sample run. The advantage of this approach is to have only **one** pretreatment file for all the samples, and the computer operates in a "smart" way to "decode" what to do with the next sample. In the case described here, preprocessing of the next pretreatment file begins 22 min after the commencement of a sample run, with the duration of autoderivatization and injection being 10 min (**Table 2**). The autosampler waits a further 4 min to inject the next sample (2 min for finishing the run and another 2 min for pump equilibration).

3.2.3. HPLC Separation System

The following chromatography conditions are highly reproducible, requiring no modification of the gradient or conditions from day to day, and only very minor modifications throughout the life of the column.

1. The preparation of mobile-phase and amino acid standard are described in detail in **Table 3**.
2. The mobile phase is a ternary solution system. Buffers are degassed with a vacuum degasser throughout the run. Salt concentration is preferably kept low in order to prolong the column life, but should be adjusted according to the resolution of the separation (*see* **Note 13**).
3. The stationary phase is a 5-μm Hypersil C18, which can be easily and simply maintained. With this column, backpressure during the run is 6–7 MPa. It is always washed with a complete gradient program twice before running standard and samples. The column can be regenerated by subjecting the column to one reverse flow and one forward flow of a 6-m*M* ammonium phosphate gradient (pH 6.5) at a flow rate of 1.0 mL/min. We do not routinely use guard columns with our system, but prefer to use a low dead volume, 2-μm stainless-steel, in-line filter. These are easily replaced, and can be cleaned by sonicating in 10% (v/v) nitric acid followed by two or three sonicating washes in Milli-Q water (*see* **Notes 14** and **15**).
4. Flow rate is kept at 1.0 mL/min. The gradient programs are shown in **Table 4**. A typical separation of amino acid standard is showing in **Fig. 3**.

Table 3
Preparation for Mobile-Phase Solutions and Amino Acid Standard

Mobile phase A[a]—30 mM ammonium phosphate (pH 6.5) in mobile phase B
Stock solutions
  2 M $(NH_4)_2HPO_4$ solution: into 100-mL volumetric flask add 26.42 g $(NH_4)_2HPO_4$
  and dilute to volume with H_2O.
  2 M $NH_4H_2PO_4$ solution: into 100-mL volumetric flask add 23.0 g $NH_4H_2PO_4$ and
  dilute to volume with H_2O.
  2 M phosphate buffer (pH 6.5): transfer the 2 M $(NH_4)_2HPO_4$ solution into 250-mL
  bottle on the magnetic stirrer, use 2 M $NH_4H_2PO_4$ solution (~ 60 mL) to adjust pH
  to 6.5, filter the solution through a 0.45-µm PVDF filter, and store it at 4°C for up
  to 6 mo.
Working mobile phase A solution
  Take 2 M phosphate stock buffer out of refrigerator, and let it reach room tempera-
  ture.
  Into a 1000-mL volumetric flask add 15 mL stock buffer and dilute to volume with
  mobile phase B.
Mobile phase B[b]
  Individually measure 850 mL H_2O and 150 mL of methanol, and mix them thor-
  oughly.
Mobile phase C
  Individually measure 900 mL acetonitrile and 100 mL H_2O, and mix them thoroughly
Amino acid standards[c]
  Internal standard solution—10 mM hydroxyproline: Into a 10-mL volumetric flask
  add 13.1 mg hydroxyproline and dilute to volume with 0.1 N HCl.
  100 µM amino acid standards: mix 20 µL 2.5 mM amino acid standard, 5 µL 10 mM
  hydroxyproline, and 475 µL borate buffer.

  [a]Mobile phase solutions are being degassed by a degasser throughout the run. We do not filter
the mobile phases in order to avoid an extra step of possible contamination. All solvents are of
HPLC-grade, and Milli-Q water is freshly obtained.
  [b]To ensure the reproducible retention time, it is necessary to measure the solvents individu-
ally. **Do not** dilute the solvent to the volume with another solvent.
  [c]For the concentration of 100 µM amino acid standard, injection of 10 µL of derivatized
mixture solution gives final on-column concentration of 250 pmol.

3.2.4. Data Analysis

Chromatography data are analyzed using the GBC WinChrom software
package before exporting the data to a spreadsheet program (Microsoft Excel).
The WinChrom software can automatically integrate the chromatogram and
assign peaks, but it is necessary always to check computer peak assignments,
and the fine detail of the type of integration used by the program. Often the
low-yield amino acids His, Tyr, and Met are difficult to integrate. We prefer-
entially use drop baseline integration rather than valley-to-valley integration,

Table 4
Composition of Three Concentrations of Ammonium Phosphate
Gradient Buffer (Buffer A = 30 m*M* Ammonium Phosphate,
pH 6.5 in Buffer B; Buffer B = 85% Methanol/15% Water [v/v];
Buffer C = 90% Acetonitrile/10% Water [v/v])

Time, min	7 m*M* salt			6 m*M* salt			5 m*M* salt		
	A%	B%	C%	A%	B%	C%	A%	B%	C%
0	19.8	65.2	15	17	68	15	14.2	70.8	15
1	19.8	65.2	15	17	68	15	14.2	70.8	15
32	12	40	48	10.8	43.2	46	9	45	46
32.05	0	0	100	0	0	100	0	0	100
34	0	0	100	0	0	100	0	0	100
34.05	19.8	65.2	15	17	68	15	14.2	70.8	15
45	19.8	65.2	15	17	68	15	14.2	70.8	15
45.1	0	100	0	0	100	0	0	100	0
55	0	100	0	0	100	0	0	100	0
55.1	0	100	0	0	100	0	0	100	0

since this gives better results throughout the life of the column. We also remove any contaminating shoulders from amino acid peaks in order to maximize the accuracy of the amino acid quantitation. Peak area data are exported to a spreadsheet program, where it is quantitated against standard amino acid mixtures. This quantitation is always done in "batches," so that amino acid data from hydrolysates are quantitated only against the standard amino acid mixture that was derivatized and chromatographed as part of that batch. Results of analysis are finally expressed as %pmol to be used for matching against the SWISS-PROT database for identification purposes. We require three parameters, which are total pmol, %pmol; and ≈pmol protein (estimated from apparent mass) (shown in **Table 5**).

4. Notes

1. Sample preparation is an important first step in amino acid analysis. Ideally, samples should be lyophilized and be free of salts, amines, and detergents. Protein separation and purification can be achieved using either 1-D or 2-D gel electrophoresis. Special attention must be given to a number of aspects when samples are to be analyzed using amino acid analysis.
   a. Milli-Q purified water or equivalent must be used throughout the electrophoresis.
   b. Proteins should be electroblotted from gels onto PVDF membranes. This chemically inert membrane is stable under the strong acid and high temperature conditions of the hydrolysis stage. Nitrocellulose membrane is degraded

Table 5
Typical Data Analysis to Be Used for Matching Against
the SWISS-PROT Database for Identification Purposes

Amino acid	125 pmol std	BSA	pmols[b]	Total pmols[c]	%[d]
D	339361280	129187760	48	190	9.5
E	337805184	195972144	73	290	14.5
S	330994112	81019424	31	122	6.1
H	210067056	11682392	7	28	1.4
G	342470368	65886344	24	96	4.8
T	320460032	83281592	32	130	6.5
A	327356256	114462208	44	175	8.8
P	355866336	79779424	28	112	5.6
Y	137513616	19983346	18	73	3.6
R	316385760	73084128	29	115	15.8
V	319988160	86532488	34	135	6.8
M	220506672	6466296	4	15	0.7
I	331897888	56424144	21	85	4.3
L	312928960	97898656	39	156	7.8
F	304652288	60422840	25	99	5.0
K	357645472	123043576	43	172	8.6
Total			499	1,994	100.0

Apparent mass = 66 kKa, ≈pmol of protein[e] = 3.5

Amino acid	125 pmol std	cox5_dicdi[f]	pmols	Total pmols	%
D	253000000	82753696	41	09	7.1
E	229000000	120000000	66	175	11.4
S	227000000	89484584	49	131	8.6
H	166000000	23508600	18	47	3.1
G	232000000	101000000	54	145	9.5
T	218000000	38773336	22	59	3.9
A	228000000	67734056	37	99	6.5
P	233000000	52492368	28	75	4.9
Y	110000000	8527441	10	26	1.7
R	213000000	30964956	18	48	3.2
V	214000000	69794608	41	109	7.1
M	150000000	9139575	8	20	1.3
I	214000000	55142756	32	86	5.6
L	214000000	89231792	52	139	9.1
F	216000000	75907184	44	117	7.6
K	275000000	122000000	55	148	9.6
Total			575	1,534	100.0

Apparent mass = 12 kKa, ≈pmol of protein = 15

[a]Three parameters can be calculated including total pmol (of amino acids); %pmol, and ≈pmol protein (estimated from apparent mass).

[b]pmol of sample amino acid = (sample peak area/standard peak area) × (pmol of standard).

[c]Total pmol = pmol of sample amino acid x dilution factor (=4).

[d]%pmol = pmol of sample amino acid/total pmol of total amino acids × 100%.

[e]≈pmol of protein = total pmols of total amino acids/total number of amino acids, where total amino acids can be calculated from the protein apparent mass divided by 115 (approximate molecular mass of amino acid).

[f]This protein was isolated from *Dictyostelium discoideum*, separated by 2-D PAGE, electroblotted onto PVDF, and identified as cytochrome-c oxidase polypeptide V (cox5_dicdi) using amino acid compositional analysis *(13)*.

      by acid, and the polyacrylamide gel pieces can not be hydrolysed directly, because acrylamide breakdown products interfere with the chromatography.

    c. To minimize glycine contamination, non–glycine-containing blotting buffer is highly desirable for electroblotting proteins from gel to PVDF membrane. Tris buffer (anode and cathode) is commonly used in semidry blotting, while CAPS buffer is suitable for wet and semidry blotting (*see* Chapter 35).

    d. PVDF membrane should be stained preferentially with amido black, although Ponceau S and Coomassie blue are also possible. After staining, the PVDF membrane should be washed extensively and then dried.

    e. Spots or bands of interest are cut using disposable scalpel blades. Make sure the area to be cut is as near as possible to a given spot or band in order to reduce the amount of PVDF membrane not carrying protein.

    f. Proteins can be eluted from the gel using passive elution buffer and transferred onto PVDF membrane by centrifugation (for example, Prosorb PE-ABD, Perkin Elmer, Cat. No. 401950). Extensive washes must be performed to eliminate salts and detergent.

2. Proteins electroblotted onto PVDF are hydrolyzed in the gas phase, so the HCl and the phenol are not in the autosampler tubes with the PVDF, but are in the bottom of the hydrolysis vessel.

3. During the evacuation and argon flush, the bottom of the vessel will become cold. The acid will boil under vacuum. Always wear safety goggles and gloves that have been washed free of talcum powder.

4. The hydrolysis described here was carried out at 160°C for 1 h. It is suitable for most of the PVDF-blotted proteins. However, for highly glycosylated proteins, i.e., mucins, more satisfactory results are obtained using a lower temperature (110°C) and a longer time (22–24 h). Lower temperature prevents the sugars on the protein from being caramelized. Also the longer hydrolysis time gives more accurate values for alanine, valine, and isoleucine.

5. Following hydrolysis, the vessel should be opened as quickly as possible to reduce the level of acid condensation inside the vessel or sample vials. A cloud of HCl vapor should be released from the vessel. Handling the vessel requires heat-resistant gloves, and safety goggles must be worn at all times.

6. If the protein spot is stained with amido black, the loss of color should be observed (not with coomassie blue or Ponceau S) following a successful hydrolysis. Also, the yellow print on the outside wall of the Chromacol vials should now be white.

7. It can be difficult to troubleshoot problems during hydrolysis, and as such, they may not become apparent until the hydrolysate is derivatized and run. The indications for a successful hydrolysis are described above. However, occasionally the analysis is completed when the indications are not clear. The first step in troubleshooting hydrolysis problems is to check that the derivatization chemistry is working correctly for the standard amino acid mixture (*see* **Note 9**). If the chemistry is working correctly, there are two further problems that have been encountered in the hydrolysis relating to defective seals.

    a. Low recovery of Met and His. This problem is only seen with protein hydrolysates and is owing to an incomplete seal allowing oxygen penetration

during the hydrolysis. The incomplete seal can arise from poor evacuation or a defective o-ring seal. Evacuating the vessel and leaving it to stand for a few minutes at room temperature can identify defective seals. If the seal is defective, it will not keep a vacuum.

    b. A variety of unknown and unusual peaks appear in the chromatography in conjunction with a general low yield of most amino acids. This is owing to a loss of acid vapor during the hydrolysis. The seal is defective and must be replaced. The unusual peaks are peptides that have been generated from the partial hydrolysis.

  8. The needle for removing the PVDF membrane must be stainless steel, such as 23-gage hypodermic. Plated needles or forceps can lead to a heavy metal contamination, which prohibits the derivatization of several amino acids, such as Lys, His, and Asp.

  9. There are a few problems that we have experienced with the derivatization chemistry, all of which are easily solved once the problem is diagnosed. The derivatization problems usually fall into two main groups: problems with derivatization chemistry itself are described below and problems with hydrolysis are outlined above.

10. Low derivatization efficiency of all amino acids indicated by the peak area and height of a standard being much lower than what is normally seen. This problem usually arises from the use of an old Fmoc solution. A fresh Fmoc solution must be made every week and should be stored at 4°C.

11. Low derivatization efficiency of amino acids His and Tyr indicated by low peak areas, whereas the areas of all other amino acids are normal. This problem is a result of inefficient conversion of di-Fmoc-substituted His and Tyr to the mono-substituted forms. This arises when the cleavage reagent is not fresh. As is normal practice, the sodium hydroxide solutions must be stored in a plastic bottle, since long-term storage in glass can lead to a release of silicates into the NaOH solution, which interferes with the derivatization.

12. Low derivatization efficiency of amino acids Asp and Glu indicated by low peak areas, whereas the other amino acids are normal. This is a difficult problem to diagnose, and essentially arises from a combination of improper borate buffer pH (too low) and ineffective mixing of the Fmoc solution during the first step of derivatization. The acidic amino acids are the slowest to be derivatized under all conditions, and if the pH of the solution is <8.6, then the derivatization reaction will proceed at a suboptimal rate. We have also found that the autosampler must manipulate volumes of no <10 μL. It appears difficult for the autosampler to mix effectively volumes of 5 + 5 μL, compared to 10 + 10 μL.

13. The most common problem is likely to arise from the coelution of His with Gly and a late-eluting Arg, possibly coeluting with Val. This can be altered by a slight (1 m*M*) increase in the ammonium phosphate concentration throughout the entire gradient. The converse of this problem will be when His coelutes with Ser, and Arg elutes close to Tyr. A slight decrease in the ammonium phosphate concentration across the entire gradient will fix this.

14. A problem often encountered is the coelution of junk peaks with amino acids. The best approach here is to eliminate the contaminant rather than trying to adjust the gradient to resolve it. Loss of Asp, His, and Lys is caused by a specific contamination of a heavy metal, and the source must be identified and removed.

15. With the extensive use of this amino acid analysis system, column performance can decline at what seems to be an unnecessarily rapid rate, showing peak broadening and loss of resolution. To keep the chromatographic separation running optimally for the life of the column, it is necessary to do a reverse wash of the column (running the entire separation gradient) every 150 or 200 injections. Although a reverse column wash with acetonitrile is not sufficient to remove all contaminating material from the column, we usually obtain about 800 runs from each column using this procedure.

Acknowledgments

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48

N-Terminal Amino Acid Sequencing of 2-DE Spots

Masaharu Kamo and Akira Tsugita

1. Introduction

Two-dimensional electrophoresis (2-DE) is one of the most advanced techniques for direct separation of considerable numbers of proteins. Direct microsequence analysis of the amino (N)-terminal partial sequence of the electroblotted protein spots from the 2-DE gels and the homology search against protein sequence database(s) have provided identification of proteins in addition to the conventional identification processes, such as comigration.

A considerably high portions of proteins have blocked N-termini that are inaccessible by the Edman degradation procedure. The blockage typically occurs with formyl, acetyl, or groups of pyrrolidone carboxyl formed by cyclization of glutamine and myristoyl group on N-terminal glycine. Nearly a half of the plant proteins are observed to be blocked at the N-termini *(1,2)*.

We developed chemical deblocking methods for formyl, pyroglutamyl, and acetyl-seryl/threonyl proteins *(3,4)*.

2. Materials
2.1. Equipment

1. Electroblotting apparatus: semidry blotting system, 20 × 20 cm equipped with a constant system maximum current of 500 mA (Nihon-eido, Tokyo, Japan).
2. Polyvinylidene difluoride (PVDF) membrane: Fluorotrans (Pall Ultrafine Filtration, NY).
3. Filter paper: Whatman 3MM.
4. Water bath: –5, 20, and 50°C.
5. Vacuum hydrolysis tube: (a) small glass tubes (4 × 40 mm) and (b) large glass tubes (13 × 130 mm).
6. Eppendorf tubes and tips: All tubes and tips are siliconized by dimethyldichrolosilane or coated by PEG4000 to avoid the adsorption of microamounts of proteins.
7. Two vacuum pumps: one for the general use and another for the hydrazine desiccator.

8. Vacuum desiccator.
9. Hydrazine vacuum desiccator: This desiccator is used only for drying hydrazine but not for general purpose. It contains concentrated H_2SO_4.
10. Protein sequencer: model 477A (Applied Biosystems, division of Perkin Elmer).

2.2. Reagents

1. Electroblotting transfer buffer: 10 m*M* 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS, Sigma) buffer containing 10% methanol, adjusted pH 11.0 *(5)*.
2. CBB staining solution: 0.1% Commassie brilliant blue (CBB, Sigma) in 50% (v/v) methanol aqueous solution *(2)*.
3. Destaining solution: 10% acetic acid and 40% (v/v) methanol aqueous solution.
4. Anhydrous hydrazine (Pierce).
5. Perfluoric acid solution: 75% (v/v) heptafluorobutyric acid (HFBA, Sigma) aqueous solution containing 5% dithiothreitol (DTT).
6. SPITC reaction solution: 1 *M* pyridine-acetate buffer, adjusted pH 6.5, containing 0.5% (w/v) 4-sulfonylphenyl isothiocyanate (SPITC, Pierce).
7. Reagents for protein sequencer (Applied Biosystems, division of Perkin Elmer).

3. Methods

3.1. Electroblotting from Gel (see *Chapter 35*)

1. Cut 14 sheets of filter papers to 20 × 20 cm size.
2. Cut a PVDF membrane to 20 × 20 cm size, and soak in methanol (at least 100 mL) for 10 min.
3. Soak the PVDF membrane and the 14 pieces of filter papers in the electroblotting transfer buffer (at least 400 mL) for 20 min.
4. Subject all the filter papers to a prerun with 100 mA for 30 min using the electroblotting apparatus.
5. Discard the bottom and top sheet.
6. Cut the 2-DE gel to 20 × 20 cm size, and rinse with transfer buffer for 5 min.
7. Set up the filter papers and membrane filters in the following order in the electroblotting apparatus; anode, 6 sheets of filter paper, the PVDF membrane, the gel, 6 sheets of the filter papers and cathode (*see* **Note 1**).
8. Carry out electroblotting with 1 mA/cm^2 for 3 h at room temperature.
9. Take out the PVDF membrane, and rinse with the electroblotting buffer.
10. Stain the membrane for 1–2 min with the CBB staining solution.
11. Destain the membrane with the destaining solution.
12. Wash the membrane with deionized water, and dry in a vacuum desiccator.
13. Store the dried membrane at –20°C.

3.2. Sequencing of Protein

1. Cut out the protein spot on the PVDF membrane.
2. Place the membrane piece in a clean Eppendorf tube, and cover with 80% methanol. Vortex the tube for 1–2 min (*see* **Note 2**).
3. Discard the methanol solution.
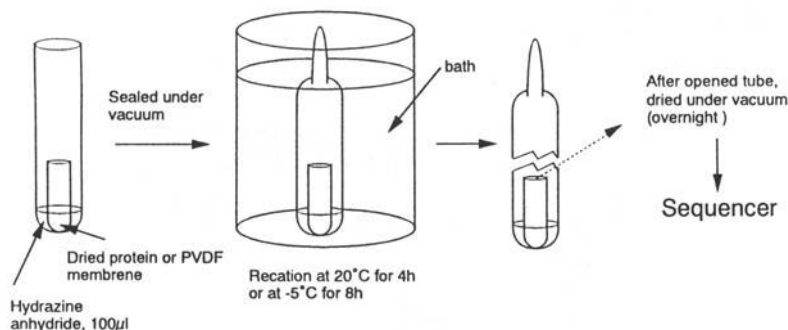
Fig. 1. N-terminal deblocking method of protein with hydrazine anhydride.

4. Carry out N-terminal sequencing for 15 cycles according to the normal program for the protein sequencer (*see* **Note 3**).
5. If a protein sequence is not detected, take the PVDF membrane piece out from the sequencer and subject to the deblocking procedure(s).

3.3. Deblocking of N-Terminal Group with Hydrazine (see Note 6)

1. Place the PVDF membrane in a small glass tube (*see* **Note 4**).
2. Dry the membrane in a vacuum desiccator.
3. Vacuum-seal the small glass tube in a large glass tube that contains 100 μL of anhydrous hydrazine.
4. After a reaction for 8 h at –5°C (*see* **Note 5**), transfer the membrane to a hydrazine vacuum desiccator, and dry over concentrated H_2SO_4 overnight (*see* **Note 6**). These procedures are shown in **Fig. 1**.
5. Return the membrane to the sequencer and carry out conventional sequencing (*see* **Subheading 3.2.**).
6. If no sequence information is obtained, continue the hydrazine reaction for another 4 h at 20°C, and repeat the conventional N-terminal sequencing methods (*see* **Subheading 3.2.**).

3.4. Deblocking of N-Terminal Group with Perfluoric Acid (see Note 8)

3.4.1. SPITC Method

1. Place the PVDF membrane in a small glass tube (*see* **Note 4**).
2. Dry the membrane in a vacuum desiccator.
3. Vacuum-seal the tube in a large glass tube that contains 100 μL of 75% perfluoric acid solution (*see* **Note 7**).
4. Incubate the sealed tube at 50°C in the bath for 1 h. Open the large tube, and transfer the small tube to the desiccator and vacuum dry (*see* **Fig. 1**).
5. Remove the membrane from the small glass tube, soak in 1 *M* pyridine acetate buffer at pH 6.5, and add SPITC solution at 50°C for 1 h.
6. Transfer the membrane to methanol, and wash several times to remove the excess of SPITC (*see* **Note 8**).

7. Transfer the membrane to the sequencer to carry out the conventional sequencing methods.

3.4.2. PITC in Sequencer Method

1. Place 75% perfluoric acid solution into the extra bottle of the sequencer (*see* **Note 9**).
2. Add a new reaction program for the first cycle to the sequencing method. The program for the ABI 477 model sequencer is listed in **Table 1**. The sequence schedule for the sequencer is shown in **Table 2**.
3. Carry out the conventional sequencing. For the first residue, the yield of PTH-Ser or PTH-Thr is low, because the amino acid residue is converted to *N*-PTH-*O*-acetyl Ser/Thr.

3.5. Homology Search

1. Access the CD-ROM "Atlas" or Network "NCBI" database (*see* **Note 10**).
2. Carry out homology search in Atlas for sequences that are <15 residues using the search program "Match" and for sequences that are >15 residues using the search program "FASTP" (PIR-International).
3. Homology search in NCBI is carried out using the "BLAST" search program.

4. Notes

1. The electroblotting procedure is performed using a semidry apparatus. To promote the blotting yield, the following caution(s) may help.
   a. Lower the methanol concentration.
   b. Raise the SDS concentration (0.01–0.02%).
   c. Reduce the polyacrylamide concentration in the gel.
   d. Remove air between PVDF membrane and gel.
   Close contact of the membrane and the gel improves the blotting yield.
2. For sequencing, we recommend washing out the CBB dye on the membrane with methanol. The dye may block the lines in the sequencer.
3. For sequencing with PVDF membrane, the sequence yield is lowered by the overlap of the membranes. The initial sequence yield also depends on protein concentration per membrane area.
4. Glass tubes are washed by nitric acid solution, that is, one-to-one dilution of concentrated nitric acid (containing 60–62% nitric acid) to incubate for overnight, and then washed distilled water.
5. In the deblocking with hydrazine at –5°C, propanol is used as the refrigerant.
6. The first deblocking reaction removes the formyl group, and the latter reaction opens the pyroglutamyl group into the γ-hydrazine derivative.
7. Do not use the desiccator and pump mixed for hydrazine and perfluoric acid. It is recommended to use two desiccators and two pumps, because a mixing of hydrazine with acid makes an insoluble salt in oil and damages pump.
8. *N*-Acetyl groups of serine/threonine residue is deblocked with concentrated perfluoric acid solution in which N-terminal acetyl group shifts to an OH group of serine/threonine. This shift reaction is faster than cleavage of *O*-ester bond.

Table 1
Reaction Program List of Acetyl Ser/Thr Deblocking Reaction[a]

Step function	Fxn no.	Values	Elapsed time
1 Reaction Heater	32	54	0 min 1 s
2 Argon Dry	29	120	2 min 1 s
3 Prep X1	10	2	2 min 3 s
4 Load X1	12	15	2 min 19 s
5 Argon Dry	29	4	2 min 23 s
6 Load S1	15	10	2 min 33 s
7 Block Flush	30	10	2 min 43 s
8 Pause	33	900	17 min 43 s
9 Pause	33	900	32 min 43 s
10 Pause	33	900	47 min 43 s
11 Load S2	18	10	47 min 53 s
12 Block Flush	18	10	49 min 3 s
13 Argon Dry	30	300	53 min 3 s
14 Prep S3	29	6	53 min 9 s
15 Deliver S1	19	12	53 min 21 s
16 Deliver S3	20	100	55 min 1 s
17 Argon Dry	30	60	56 min 1 s
18 Deliver S1	14	12	56 min 13 s
19 Deliver S3	20	100	57 min 53 s
20 Pause	33	30	59 min 23 s
21 Deliver S3	20	100	60 min 3 s
22 Argon Dry	29	180	63 min 3 s
23 Prep R1	1	6	63 min 9 s
24 Deliver R1	2	2	63 min 11 s
25 Argon Dry	29	2	63 min 13 s
26 Pause	33	120	65 min 13 s
27 Prep R1	1	6	65 min 19 s
28 Deliver R1	2	2	65 min 21 s
29 Argon Dry	29	40	66 min 1 s
30 Prep R2	4	6	66 min 7 s
31 Deliver R2	5	120	68 min 7 s
32 Prep R1	1	6	68 min 13 s
33 Deliver R1	2	2	68 min 15 s
34 Argon Dry	29	40	68 min 55 s
35 Deliver R2	5	400	75 min 35 s
36 Prep R1	1	6	75 min 41 s
37 Deliver R1	2	2	75 min 43 s
38 Argon Dry	29	40	76 min 23 s
39 Deliver R2	5	400	83 min 3 s
40 Prep R1	1	6	83 min 9 s
41 Deliver R1	2	2	83 min 11 s
42 Argon Dry	29	40	83 min 51 s
43 Deliver R2	5	400	90 min 31 s
44 Reaction Heater	32	48	90 min 32 s
45 Argon Dry	29	180	93 min 32 s
46 Deliver S1	14	120	95 min 32 s
47 Deliver S2	17	240	99 min 32 s
48 Argon Dry	29	120	101 min 32 s

[a]Sequencer was used Perkin Elmer, ABI 477A model.

This program was used for Reaction Cycle of 477A model. Cycle length is 49 steps and run time is 101 min 32 s.

X1: 75% Heptafluorobutyric acid/water contains 0.2% DTT.

Table 2
Sequencer Schedule of ABI 477A Sequencer

Cycle no.	Reaction cycle	Conversion cycle
1	Block-1	Begin-1
2	Begin-1	Begin-1
3	Normal-1	Normal-1
4	Normal-1	Normal-1
—	—	—
—	—	—

After drying sample to remove the acid, the coupling reaction with SPITC was achieved at pH 6.5. After the couple reaction, the protein was removed from the reagent and directly analyzed with the sequencer.

9. The sequence program in the first cycle is changed to react 75% HFBA solution for 45 min at 50°C. After the $N{\rightarrow}O$ shift of acetyl group has taken place, an excess amounts of PITC is added and then the pH is gradually raised by adding trimethylamine. Before the reverse $O{\rightarrow}N$ shift takes place at an alkaline pH, PITC may reacts with the exposed α amino groups. After the first cycle, the normal reaction program is continued.

10. CD-ROM of "Atlas" program is obtained from PIR-International and is distributed in the Americans by the National Biomedical Research Foundation (TEL: 1-202-687-2121, e-mail: PIRMAIL@GUNBRF.BITNET), in Europe by Matinsried Institute for Protein Sequence (tel: 49-89-8578-2657, e-mail: meves@ehpmic.mips.biochem.mpg.de), and in Asia and Oceania by Japan International Protein Information Database (tel: 81-471-23-9778, e-mail: tsugita@jipdalph.rb.noda.sut.ac.jp).

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49

Characterizing Proteins from 2-DE Gels by Internal Sequence Analysis of Peptide Fragments

Strategies for Microsample Handling

Hediye Erdjument-Bromage, Mary Lui, Lynne Lacomis, and Paul Tempst

1. Introduction

For years, proteolytic digest and peptide purification were essential tools for the biochemist wishing to determine the primary structure of a protein. The strategies changed with the availability of a polypeptide gas-phase sequencer and the advent of DNA recombinant technology. However, blocked N-termini and changing trends in molecular cloning techniques, such as PCR, brought back "internal" sequencing as well, a term coined by Aebersold et al. in their seminal paper on the *in situ* micropreparative digestion of electroblotted proteins *(1)*. Since then, many practical improvements of the original digest recipe and alternative approaches have been proposed *(2)*. Considerable effort has also been expended to interface *in situ* digestion with micro-liquid chromotography (LC), peptide sequencers, and mass spectrometers. The ability to generate a set of specific peptides (e.g., tryptic), together with recent advances in biopolymer mass spectrometry provide a novel approach to protein identification. Accurate masses of several protein fragments compose a "peptide mass fingerprint," theoretically sufficient for unambiguous searching of sequence repositories *(3)*. It is therefore expected that enzymatic proteolysis will become even more widely used in the future.

With strategies for protein chemical characterization and identification now well-established, enhanced sensitivity will likely become the focus of further developments. To that end, major efforts have been made, at great expense, to improve chromatographic and analytical devices. Less attention has been directed at optimizing the "connecting" steps of the process, namely sample preparation and

handling. In this chapter, we describe our current protocol for fragmentation of proteins bound to nitrocellulose or PVDF *(4)*. The presence of Zwittergent 3-16 as an additive for one-step enzymatic digestion is a critical element, both in terms of high-yield recovery and general applicability of this procedure. After digestion, the disulfide bonds are reduced using β-mercaptoethanol and the cysteine residues are S-alkylated with 4-vinylpyridine. The derivatized cysteine residues are easily detected during chemical sequencing. The digest protocol that we describe is compatible with most downstream chromatographic and analytical procedures.

2. Materials
2.1. Reagents

1. Digest buffer: 1% Zwittergent 3-16 in 0.1 *M* ammonium bicarbonate. Weigh 10 mg Zwittergent 3-16 (Calbiochem; kept as powder form at –20°C). Add 500 μL Milli-Q water, and warm the solution at 37°C for 10 min or until completely dissolved. Add 500 μL 0.2 *M* ammonium bicarbonate solution.
2. Ammonium bicarbonate stock solution: 0.2 *M* ammonium bicarbonate. 0.79 g of ammonium bicarbonate in a total volume of 50 mL of Milli-Q water. Store at 4°C.
3. Reducing reagent: 1% β-mercaptoethanol, 990 μL water + 10 μL β-ME (in refrigerator). Vortex. This is done in the hood. This reagent is made fresh each day.
4. Alkylating reagent: 10% 4-vinylpyridine. 90 μL absolute ethanol + 10 μL 4-vinylpyridine (both kept in explosion-proof freezer). This is done in the hood. This reagent is made fresh each day.

2.2. Equipment

1. Microbore HPLC systems as described in Tempst et al. *(5)* and Elicone et al. *(6)*:
   a. 140B Solvent Delivery System equipped with a 75-μL dynamic mixer (both from Perkin Elmer [PE] Applied Biosystems).
   b. Rheodyne 7125 injector (Rainin) fitted with a 50-μL loop.
   c. 783 detector (PE Applied Biosystems).
   d. U-Z view flow cell (LC Packings).
   e. Vydac C18, Reliasil ODS, Inertsil C18 colums, all 1 × 150 mm, have been used successfully.
   f. Kip and Zonen strip chart recorder.
   g. HPLC signals are sent to an A/D converter (PE Nelson), and chromatograms analyzed and plotted using PE Nelson Turbochrom 4 software.
2. REFLEX III MALDI reflectron-TOF instrument (Bruker-Franzen).
3. PE Applied Biosystems Model 477A automated sequenator as described in Tempst et al. *(7)*.

3. Methods
3.1. Sample Requirement

After electrophoresis, the 2-DE gel is electrotransferred to nitrocellulose (NC) support (*see* Chapter 35). It is preferable to view the entire piece of mem-

brane onto which the sample gel has been electroblotted. This allows a clear visual assessment of the real "background" or the unspecific staining of membrane at the same time as the staining of protein to be characterized.

At this point, the spot of interest is stained first with Ponceau S and, if necessary, followed by Amido Black: spots are then carefully cut and excess membranes trimmed away (*see* Chapter 36). An area of membrane, away from the protein lanes, is also excised and used as a protease control (blank) for the *in situ* digest. Before storage, the precise size of the membrane, as well as the staining intensity of the protein on the membrane, are noted. Excised membranes can be kept dry, in freezer, until ready to be processed.

3.2. HPLC Preparation

1. Run the HPLC isocratic overnight (90% B; 10 μL/min for 1- and 0.8-mm systems) a day before the *in situ* digest is done.
2. On the day of the digest, run one or more HPLC reagent blanks until the baseline and background are acceptable.
3. Run a trypsinized cytochrome-*c* peptide mix (5 pmol for 1- and 0.8-mm HPLC systems) to check the performance of the HPLC system.
4. If the cytochrome-*c* is okay, make the HPLC ready for the run.

3.3. In Situ *Proteolysis*

1. While keeping the nitrocellulose damp in a clean petri dish, cut the NC into 1-mm^2 pieces; note that if NC appears "staticky," it is drying out.
2. Once diced, transfer the NC pieces into a clean, labeled microcentrifuge (0.5-mL size) tube containing 5–25 μL of digest buffer. The digest volume varies with the surface area of the membrane. The NC pieces must be superficially covered with buffer (*see* **Note 1**).
3. Add the protease, and mix the pieces with the pipet tip; do not vortex (*see* **Note 2**).
4. Place in a 37°C water bath for 2 h (*see* **Note 3**).
5. Remove the samples from the water bath, and sonicate for 5 min.
6. Centrifuge the samples for 1 min at 2000*g*. A minicentrifuge works best.
7. Extract 5–25 μL of the liquid sample, and transfer it into a new, labeled microcentrifuge tube (0.5 mL) retaining the pipet tip in this second tube. Leave the solid NC in the old tube.
8. Take an equal volume of the digest buffer, and add it to the old tube to rinse off the NC pieces. Vortex the tube, and then centrifuge at 2000*g* for 5 to 10 s. Throw out this pipet tip, and replace with the saved tip.
9. Draw up the liquid from the old tube (keep the junk NC until the end of day), and combine it that in the new tube. Note the total sample volume. Place the sample on ice immediately.
10. Prepare the reducing reagent.
11. Lightly blow argon over the top of the sample to replace the oxygen from air, and cap the tube.

12. Take 2 μL of the reducing reagent (for a 20-μL sample volume), and add it to the sample (the final concentration of β-ME is 0.1% [v/v]). Vortex lightly, and blanket with argon gas.
13. Place the sample in a 37°C water bath for 30 min.
14. Prepare the alkylating reagent.
15. After 30 min, remove the sample from water bath, and lightly blow argon over the top the sample.
16. Add 0.67 μL of the alkylating reagent to the 22 μL total digest volume (the final concentration of alkylating reagent is 0.3% [v/v]). Vortex lightly and blanket with argon gas.
17. Incubate the sample at room temperature in total darkness for 30 min (*see* **Note 4**).
18. Prepare the HPLC while the alkylating reaction is running.
19. At the end of alkylation, centrifuge the sample at 14,000*g* for 2 min using a microcentrifuge next to the HPLC system.
20. Inject the sample immediately onto the HPLC, or place it on ice (max. 10–15 min). There is no need to acidify the digest before HPLC (*see* **Note 5**).
21. Number up to 70 microcentrifuge tubes (0.5 mL) for an average-size protein (~60 kDa).
22. After the chemical peaks elute from column, wait for the HPLC baseline to settle to steady absorbance.
23. Start the HPLC gradient.
24. Hand-collect peptides into microcentrifuge tubes, and immediately place on ice.
25. Overlay the completed HPLC profile with the trypsin blank (control) that had been previously obtained (*see* **Note 5**).
26. Select 10 tryptic peptides above background. The peptides exhibiting symmetrical peaks, free of adjacent shoulders are usually picked.
27. Individually deposit a fraction of a collected peak (0.1–0.5% of peptide) on a delayed extraction MALDI-reflectron-TOF MS plate with and without added internal calibrants *(8,9)*.
28. Screen the peptide peaks for "purity," i.e., mixtures. Although the suppression of signal in mixtures of peptides arises, giving misleading information about the homogeneity of a peptide under study, this is still the best approach when determining which peptides to use for a subsequent chemical sequencing experiment.
29. Place tubes in a box labeled with the HPLC run number, and store at –70°C.
30. For chemical sequencing, add trifluoroacetic acid (up to 50% v/v) to the tube containing the peptide before loading the sample onto the sequencing disk for internal sequencing *(8,10)*.
31. Carry out internal sequencing of the peptide using an ABI 477 sequenator.

4. Notes

1. The presence of 1% Zwittergent prevents sticking of generated peptides to the polypropylene Eppendorf tube in which *in situ* digest is carried out. This eliminates the need to acidify the tube before drawing liquid for HPLC separation.
2. The choice of enzyme is trypsin. Trypsin (Promega Modified Trypsin, Sequencing Grade, stock in 1 μg/uL Milli-Q water, in –20°C) aliquots of 0.2 μg/μL are

made up for the *in situ* digest. One μL (200 ng) is typically added to the digest tube. This is approx 5 ng protease/1 mm^2 of nitrocellulose (for instance, for two pieces of NC with total of 40 mm^2 surface area, 200 ng of trypsin are added).

3. When dealing with large proteins (100 kDa and above), we are supplementing the reaction with a second aliquot of fresh trypsin after 1 h of proteolysis. This provides an advantage for database mass fingerprinting experiments, since the tryptic peptides generated have less missed cleavage sites. Interestingly, the autolytic peptides arising from the enzyme itself are not increased significantly with this supplement of fresh enzyme.

4. Get the HPLC ready to inject. Timing is very important: do not start alkylation until HPLC has been checked. The HPLC analysis must be performed immediately on the alkylated sample. Otherwise, the HPLC profile will be severely affected.

5. The trypsin blank (control) is processed and chromatographed on the same day as the sample, but since it does not need to be hand-collected, proceed with **Subheading 3.3.**, **steps 24–28** during HPLC of control.

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50

Obtaining Molecular Weights of Proteins and Their Cleavage Products by Directly Combining Gel Electrophoresis with Mass Spectrometry

Rachel R. Ogorzalek Loo, Joseph A. Loo, and Philip C. Andrews

1. Introduction

Methods combining the high throughput and sensitivity of matrix-assisted laser desorption ionization mass spectometry (MALDI-MS) with polyacrylamide gel electrophoresis (PAGE) are rapidly gaining attention, but almost exclusively for applications eluting proteolytic digest products from gels and membranes *(1–9)*. These methods fulfill a considerable need for identifying unknown proteins isolated in polyacrylamide gels, but they do not provide masses for intact proteins, a particular concern should the goal be the understanding of anomalous electrophoretic migrations, characterizing the complete ensemble of posttranslational modifications displayed by proteins, or understanding why multiple protein spots on a two-dimensional (2-D) gel are all identified as the same protein. These issues are particularly relevant for low-abundance proteins, which may be identified by matching the masses of only a few tryptic peptides to those predicted from cleavage of an unmodified protein, or by matching small stretches of sequence information *(9,10)*. In some cases, those matches may correspond to <10% of the total protein sequence *(9)*. Analysis of intact proteins has been performed following electroblotting to membranes *(11–19)*, but we will describe a method to link gel electrophoresis directly to MALDI-MS, yielding masses of both intact proteins and cleavage products without electroelution or electroblotting *(20–22)*. Key to the success of this approach are ultrathin polyacrylamide gels, which dry to thicknesses of 10 µm or less and which have the additional advantages of rapid preparation and electrophoresis run times. These methods are applied to isoelectric focus-

ing (IEF), native, and sodium dodecyl sulfate (SDS) gel electrophoresis. **Figures 1–5** show examples of MALDI-MS proteins spearated by IEF and SDS gel electrophoresis. At this point, they have been optimized for one-dimensional (1-D) separations, although they could be applied to two-dimensional (2-D) separations by following the preparation protocols for SDS gels. In addition, they make it possible to run "virtual 2-D gels" in which proteins are resolved in the first dimension on the basis of their charge (i.e., IEF gels), whereas the second dimension is MALDI-MS-measured molecular weight instead of SDS gel electrophoresis (*see* **Fig. 4**) *(22)*. The robustness of IEF gels, at least in these mass spectrometry applications, suggests that an ideal approach to obtaining information related to 2-D gel spots involves running duplicate immobilized pH gradient (IPG) gels. One is used to construct the classical 2-D gel, and one is prepared for MALDI- MS. The reproducibility of the IPG gels ensures that results from the two experiments may be compared directly.

2. Materials
2.1. Equipment
1. PhastSystem automated gel electrophoresis unit (Pharmacia, Uppsala, Sweden).
2. Precast thin polyacrylamide gels with polyester film backing (Pharmacia; *see* **Note 1**):
   a. For IEF, linear gradient, pH 3.0–9.0, carrier ampholyte gels (5% polyacrylamide, 0.35 mm thickness) are employed.
   b. For native and SDS gel electrophoresis, either homogeneous (12.5% polyacrylamide, 0.45 mm thickness) or gradient (8–25% polyacrylamide, 0.45 mm thickness) gels with 0.112 M acetate, 0.112 M Tris, pH 6.5, are employed.
3. Time-of-flight mass spectrometer, preferably equipped with time-lag focusing (delayed extraction), a large area sample stage, and a nitrogen laser for desorption at 337 nm (*see* **Note 2**).

2.2. Reagents
1. SDS mol-wt marker proteins (Pharmacia), IEF markers (Bio-Rad, Hercules, CA).
2. IEF sample buffer: Novex pH 3.0–7.0 sample buffer (Novex, San Diego, CA) diluted 1:10 with water, 0.05 mg/mL methyl red (Aldrich, Milwaukee, WI).
3. SDS sample buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2.5% SDS, 0.01% bromophenol blue, 0.55% β-mercaptoethanol.
4. Native sample buffer: Novex native sample buffer, diluted 1:10 with water.
5. Buffer strips for SDS gel electrophoresis (Pharmacia): 3% agarose, 0.2 M tricine, 0.2 M Tris-HCl, pH 8.1, 0.55% SDS.
6. Buffer strips for native gel electrophoresis (Pharmacia): 3% agarose IEF grade containing 0.88 M L-alanine and 0.25 M Tris, pH 8.8.
7. IEF fixation solution: 20% trichloroacetic acid.
8. Wash solution: 30% methanol, 10% acetic acid.

9. IEF stain solution: 0.02% Phast Gel Blue R solution (Pharmacia), or Coomassie brilliant blue R-250 or G-250 (Sigma), 30% methanol, 10% acetic acid, 0.1% $CuSO_4$.
10. Destain solution: 30% methanol, 10% acetic acid.
11. Sinapinic acid "plasticizer" solution for SDS and native gels: 0.1 g sinapinic acid in 10 mL of 50% acetic acid, 50% acetone.
12. UV matrices for mass spectrometry: saturated sinapinic acid or α-cyano-4-hydroxy cinnamic acid (Aldrich) in 50% CH_3CN, 49.9% H_2O, 0.1% trifluoroacetic acid (TFA), or 33% CH_3CN, 66.9% H_2O, 0.1% trifluoroacetic acid.
13. CNBr cleavage solution: 10 mg/mL CNBr in 50% TFA, β-mercaptoethanol.
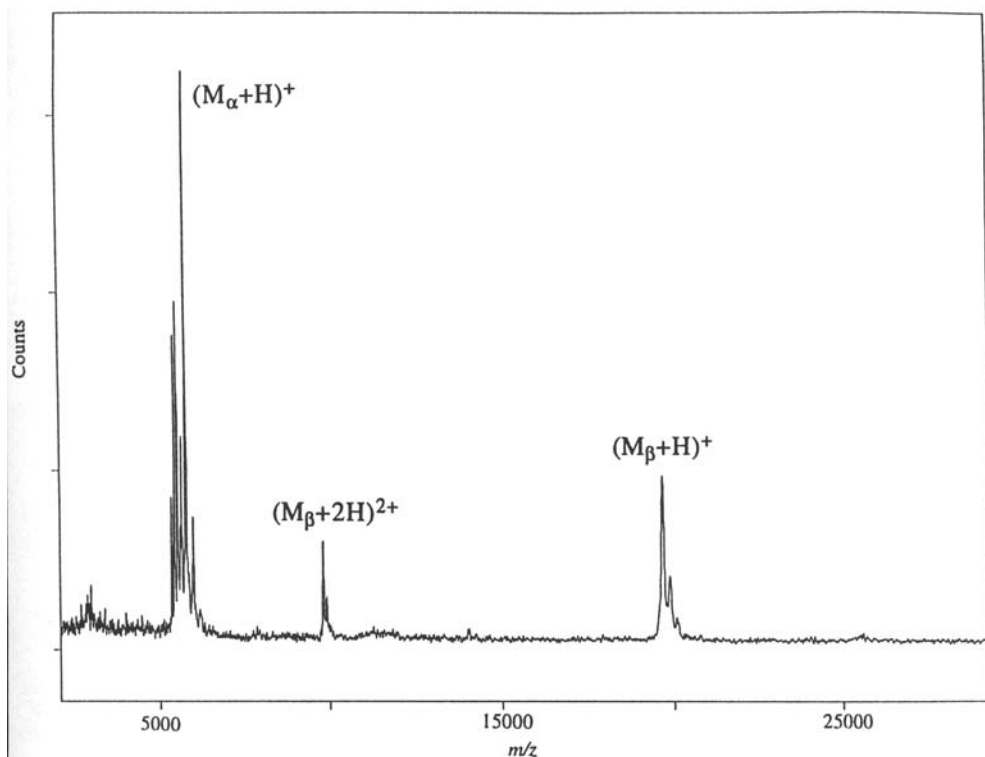
Fig. 1. MALDI mass spectrum of the middle (p*I* 8.8) lentil lectin band from an IEF gel. Ions for the α- and β-subunits are labeled. (Reprinted with permission from ref. *21*.)
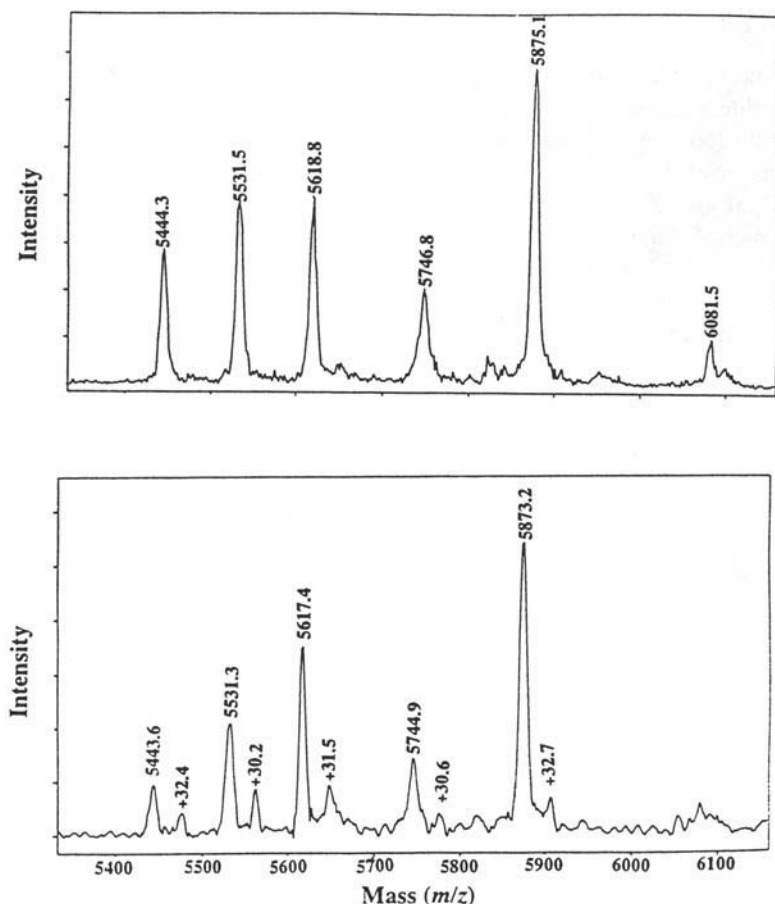
Fig. 2. Mass spectra of the α-subunit of lentil lectin obtained at laser positions separated by only a few tenths of a millimeter at the location of the p*I* 8.8 band; the top spectrum corresponds to the more basic p*I*. Heterogeneity arises from C-terminally truncated forms of this protein. (Reprinted with permission from ref. *22*.)

Fig. 3. *(opposite page)* Composite MALDI spectrum from a mixture of proteins on an IEF gel. The proteins are **(A)** bovine trypsinogen (2 pmol distributed over 2 bands), **(B)** and **(C)** bovine hemoglobin (700 fmol each α chain and β chain), **(D)** carbonic anhydrase (700 fmol), **(E)** and **(F)** soybean trypsin inhibitor (2 pmol), and **(G)** bovine albumin (2 pmol). The top of the gel corresponds to the most basic proteins. On the left is a similar gel, but stained with Coomassie blue and with protein loadings 50 times higher. (Reprinted with permission from ref. *22*.)
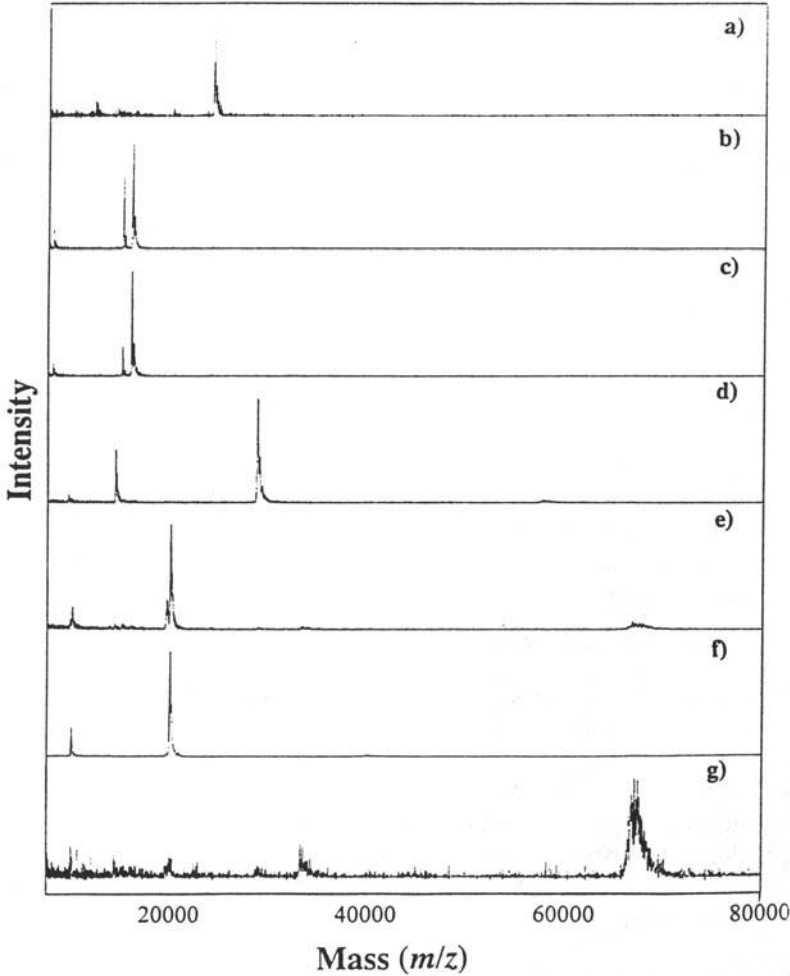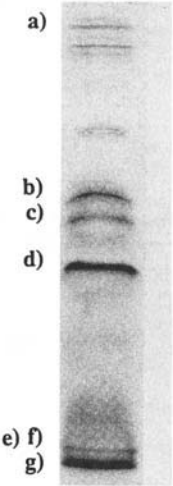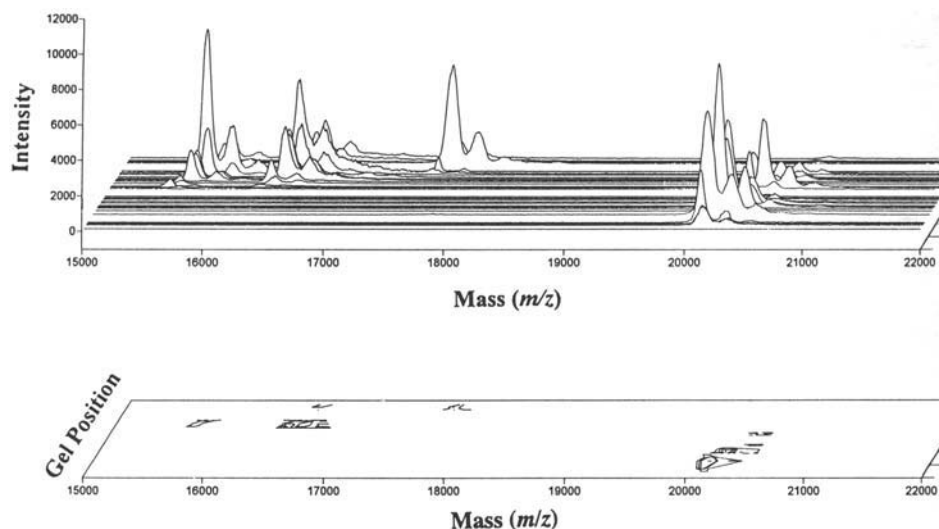
Fig. 4. 3-D plot and 2-D projection ("virtual 2-D gel") assembled from MALDI spectra obtained at various positions down 1 cm. of a single lane on an IEF gel loaded with the proteins of **Fig. 3** at 10–20 pmol in addition to myoglobin and lentil lectin.

3. Methods

3.1. Electrophoresis and Gel Preparation for Mass Spectrometry

3.1.1. Electrophoresis

1. Samples are dissolved in the appropriate buffer (IEF, native, or SDS) and loaded by dipping a comb (0.5, 1, or 4 μL capacity/lane) into the sample solutions and placing the comb on top of the gel. Because the samples are not loaded in wells, the proteins migrate primarily along the surface of the gel, making them more accessible for MALDI than proteins run on thicker gels.
2. Electrophoresis conditions for the PhastSystem *(23–25)* detailed by the manufacturer are followed, except that for IEF, the cooling temperature is reduced to 10°C.

3.1.2. Stained IEF Gels (see **Note 6**)

1. After electrophoresis, soak the gel in IEF fixation solution for 20 min.
2. Soak the gel in wash solution for 15 min.
3. Stain the gel in the Coomassie blue stain solution *(26)* (*see* **Notes 3** and **4**).
4. Destain the gel in destain solution for 15 min.
5. Dry the gel at room temperature lightly covered (*see* **Note 7**).
6. Spot the desired bands with 0.5–1 μL UV MALDI matrix.
7. Allow the matrix to dry before inserting the gel into the mass spectrometer.
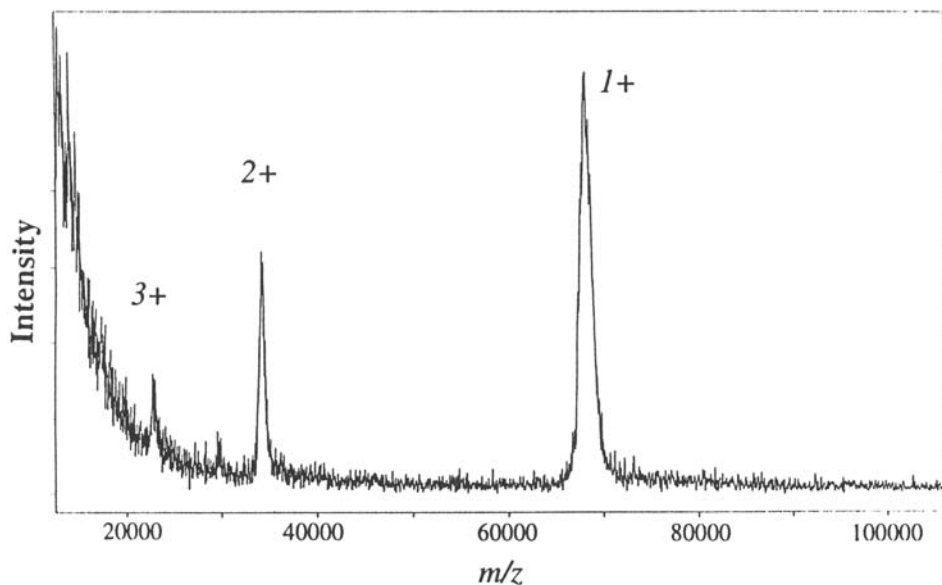
Fig. 5. Mass spectrum of bovine albumin (M_r 66430) isolated on an SDS gel. (Reprinted with permission from ref. *22*.)

3.1.3. Unstained IEF Gels (see **Note 6**)

1. After electrophoresis, the gel is soaked in IEF fixation solution for 20 min.
2. Wash the gels in two changes of IEF wash solution for 15 and 30 min each, respectively.
3. Soak the gel in the UV MALDI matrix solution for 10 min. (*See* **Note 5**.)
4. Allow the gels to dry at room temperature, at least overnight.

3.1.4. Unstained Native and SDS Gels

1. Wash native and SDS gels with wash solution for 15 min.
2. Repeat **step 1**.
3. Soak the gel in sinapinic acid plasticizer solution for 15 min.
4. Dry the lightly covered gel at room temperature.
5. Spot the gel with UV MALDI matrix (sinapinic acid). (*See* **Note 7**.)

3.2. CNBr Cleavage

1. In-gel CNBr digests *(27–29)* are performed by cutting out the band of interest from stained IEF gels and spotting 5 μL of 10 mg/mL CNBr in 50% TFA. For mass spectrometry, TFA is preferable to the formic acid traditionally employed in CNBr digest protocols, because it prevents N- and O-formylation of the products *(30,31)*.

2. Incubate the excised gel band in the dark at room temperature for 2–4 h.
3. For samples requiring *in situ* reduction, spot bands with 1 μL of β-mercaptoethanol, and incubate for 15 min.
4. Dry the sample on a centrifugal dryer.
5. CNBr products are analyzed directly from polyacrylamide gels by spotting with either sinapinic acid or α-cyano-4-hydroxy cinnamic acid. An example is shown in **Fig. 6**. This method has not been tested for analysis directly from SDS or native gels.

3.3. Mass Spectrometry

1. Gels with the polyester backing intact are trimmed at the edges to fit the MALDI sample plate and are mounted with adhesive tape. (*See* **Note 7**.) Because of the good match between the Phast gel and sample plate size on some commercial mass spectrometers, all of the lanes from a single gel can be mounted onto a single plate.
2. CNBr-digested bands from IEF gels are also taped to the sample plate.
3. Insert the sample plate into the mass spectrometer, and wait for the instrument to reach suitable pressure. Unstained gels require 5–10 min to pump down in the mass spectrometer, whereas Coomassie blue stained gels need up to 30 min. These times may vary, depending on the configuration of the mass spectrometer.
4. Analyze spots or bands by mass spectrometry. Generally data from 25–100 laser shots were averaged for each mass spectrum. (*See* **Note 8**.)

4. Notes

1. The methods described here have been applied to Pharmacia Phast gels; gels from other manufacturers or from the same manufacturer, but sold for other systems may be more or less rugged. For example, we have observed that the Pharmacia Excel gels disengage from their plastic backing on exposure to the plasticizer solution employed in our protocol for SDS and native gels, but Pharmacia Immobiline DryStrip (immobilized pH gradient) gels are compatible with our IEF preparation methods. Gel thickness, percent polyacrylamide, and method of attachment to the polyester backing are key issues affecting success.
2. The data described here were acquired with one of three time-of-flight mass spectrometers operated in linear mode:
   a. Vestec LaserTec ResearcH (1.3-m pathlength, continuous ion extraction).
   b. PerSeptive Elite (2.0-m pathlength, time-lag focusing).
   c. Elite-XL (4.2-m pathlength, continuous extraction).
   The three instruments were all fitted with nitrogen lasers (337 nm) and 100-position sample plates modified as described previously *(20–22)*. In particular, a 4 × 4-cm region of the sample plate was milled to a depth of 0.5 mm to maintain adequate spacing between the sample surface and the ion source grid. This, or a similar modification, is highly recommended for undertaking these experiments on PerSeptive delayed extraction instruments in order to minimize chances of arcing. Time-of-flight mass spectrometers from other manufacturers are also compatible with analysis directly from polyacrylamide gels.
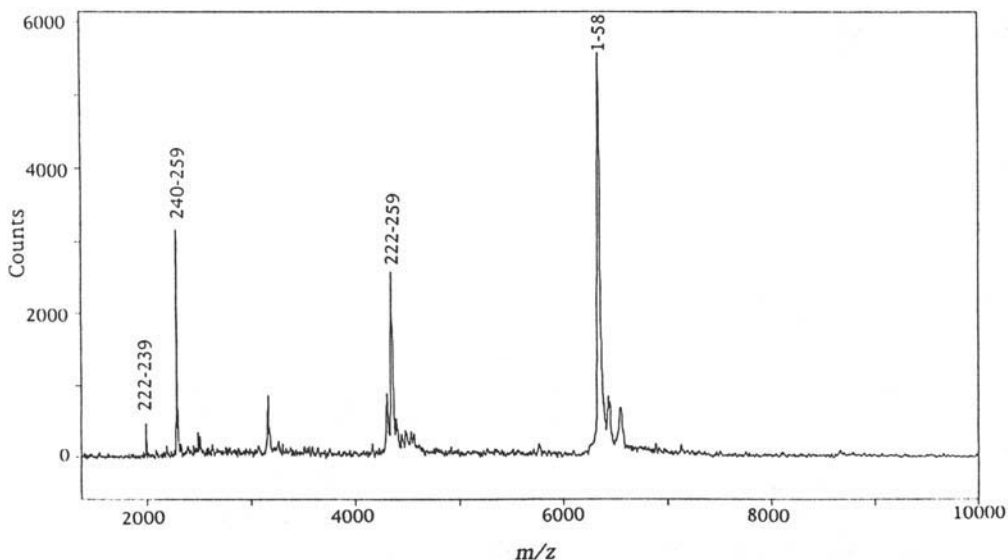
Fig. 6. MALDI mass spectrum of an in-gel CNBr digest of bovine carbonic anhydrase II, 3 pmol loaded on an IEF gel.

3. As an alternative to G-250 stain, the gel can be stained with either 0.02% PhastGel blue R solution (Pharmacia) or Coomassie brilliant Blue R-250.

4. Commonly used staining methods, although desirable for visualization and quantitation, can interfere with mass analysis owing to protein modification, adduct formation, or ion suppression. For example, Coomassie blue has been observed to yield bothersome adducts in MALDI spectra *(15,21)*, although the adducts observed in UV desorption directly from gels *(21)* were considerably reduced over those observed from desorption off membranes *(15)*. For small proteins, the 800-Dalton Coomassie adducts can be resolved *(21)* from the unmodified ions. Increased washing to remove all of the stain has avoided adduct problems for work with excised, digested bands *(9)*. Shifting to reversible stains may also be a helpful strategy, although less convenient, since the visualization will then be temporary.

5. To avoid soaking the entire gel in matrix solution, specific regions of the gel can be spotted with sinapinic acid matrix. After the 30% methanol/10% acetic acid destain, dry the gel and spot the desired bands with sinapinic acid solution.

6. Generally MALDI is performed on unstained gels in order to avoid contributions to the mass spectrum from dye adducts to the protein ions, and because unstained gels often provide better sensitivity. Protein bands can be located in advance of the mass spectrometry by comparing a duplicate, stained gel.

7. Gels are usually examined by MALDI-MS within a few days of preparation. Cracking of gels is more likely as they age and is affected by the composition of

the gel with higher percent polyacrylamide gels more prone to cracking. For desorption directly from polyacrylamide, IEF gels have the preferred properties. The IEF gels have rarely cracked and have survived multiple atmospheric pressure-to-vacuum cycles. They are stable for weeks and even months. SDS gels have been more difficult to handle, often cracking after 1 h in the mass spectrometer. Although we have not had problems with cracked gels detaching from their backing under vacuum, this possibility should always be considered. It is recommended that SDS gels be examined soon after preparation (but after complete drying) and that the cycling between atmospheric pressure and vacuum be minimized. The edges of the gel should be secured to the sample stage carefully. It is possible to continue acquiring spectra with reduced mass accuracy from gels that have cracked, and internal standards can compensate for the accuracy limitations.

8. Mass accuracy in desorption from gels is an important concern, particularly because the use of internal mass standards is much more limited than when simply desorbing samples spotted onto the mass spectrometer probe. Moreover, uneven gel thicknesses, difficulty in mounting gels flat, and surface charging affect mass accuracy in ways not encountered for more routine acquisition of mass spectra from metal surfaces. Studies examining entire gels or blots, as opposed to excised bands or spots only a few mm^2 in size, pose special challenges because an hour or more can be consumed examining a few lanes, constantly building up charge on the insulator surface. In one case with continuous ion extraction, when an entire lane was scanned based on calibration from a single spot at the top of the gel, a 1% drift in mass was observed, with the size of the drift reflecting the spatial distance from the internal standard *(22)*. In order to obtain accurate mass values when desorbing from gels and membranes in continuous extraction instruments, considerable attention must be paid to running frequent standard spots. By placing multiple calibration spots down a blank lane bordering the sample lane and calibrating with nearby standard spots, mass accuracy has been extended to 0.1–0.2% in linear mode, with continuous extraction *(22)*. The main limitation regards extension of this technique to 2-D gels, where one does not always have the luxury of applying mol-wt calibration spots anywhere one wishes. In principle, internal calibration (spotting a standard directly on top of the band of interest) would be superior, but it is particularly difficult with gels, because nonoptimal standard loadings can overwhelm sample signals. Delayed extraction overcomes many of the mass accuracy limitations experienced for desorption directly from gels, and accuracies to better than 0.1% are readily obtained. Although calibration spots can be spaced more widely with delayed extraction, they cannot be eliminated entirely for desorption directly from polyacrylamide gels.

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51

Identification of Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using Peptide and Fragment Ion Masses

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1. Introduction

Methods for the identification of proteins have advanced dramatically this decade through the introduction of mass spectrometric techniques and instrumentation sensitive enough to be applicable to biological systems *(1)*. The two mass spectrometric techniques that have provided these advantages are electrospray-ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (*see* Chapters 52, 54, and 55).

This chapter will deal with the identification of gel-separated proteins, whether or not they have been electroblotted to a PVDF-based membrane. Therefore, we will detail methods used to generate peptide fragments from in-gel digests or on-membrane digests from Immobilon-P or Immobilon-CD, using essentially the methods of Moritz et al. *(2)*, Pappin et al. *(3,4)*, and Patterson *(5)* (as modified from Zhang et al. *[6]*), respectively (*see* Fig. 1). The microcolumn sample clean-up approach is a more detailed description of that which we have published previously *(7)*. We will not reiterate the electrophoretic methods required to separate and electroblot the proteins to a membrane or visualize the proteins (*see* Chapters 35–37). MALDI-MS methods for accurate mass determination of the released peptide fragments together with approaches that utilize this peptide-mass data for identification will then be covered.

The principle behind identification of proteins via accurate mass measurements of enzymatically derived peptides relies on the frequency of specific cleavage sites within a protein yielding a set of potential peptide masses that are unique to that sequence entry when compared with all of the others in the

database. Studies have shown that only four to six peptide masses are required to identify proteins in searches of a database of more than 100,000 entries *(8)*. Masses sufficiently accurate for this purpose are easily obtained using MALDI-MS. The approach requires that the protein to be identified (or a very close sequence homolog) exists in the database. The caveats to this approach and considerations for interpretation of the results will be described.

Finally, we will cover generation and analysis of sequence-specific fragment ions using postsource decay MALDI-MS. Either partially interpreted or uninterpreted fragment ion data can be used in search programs to identify proteins with high accuracy in sequence databases, and the use of this approach will also be described.

2. Materials
2.1. Equipment

1. Water bath (able to be set at 25°C or 37°C).
2. Sonication bath.
3. Vacuum concentrator (e.g., Speed-Vac, Savant, Farmingdale, NY).
4. Polypropylene microcentrifuge tubes (500 µL).
5. MALDI-MS instrument (linear and/or reflector capability).
6. MALDI-MS instrument fitted with a reflector capable of variable voltage or a curved-field reflector (the example will employ the Kompact MALDI IV—a curved-field reflector MALDI-MS [Kratos Analytical, Ramsey, NJ]).

2.2. General Reagents

1. 50% v/v Methanol (MeOH) containing 0.1% v/v aqueous trifluoroacetic acid (TFA).
2. Deionized water (dH_2O).

2.3. Enzymatic Digestion In-Gel

1. 50 mM citric acid.
2. 20 mM NH_4HCO_3 containing 50% v/v acetonitrile (MeCN).
3. Stock enzyme: Endoproteinase LysC (5-µg vial, sequencing-grade) (Boehringer Mannheim, Indianapolis, IN) (LysC).
4. 20 mM NH_4HCO_3, 1.0 mM $CaCl_2$ (digest buffer).
5. Laboratory rotating mixer.
6. 1% TFA.
7. 60% v/v MeCN containing 0.1% v/v aqueous TFA.

2.4. Enzymatic Digestion on Immobilon-CD

1. 20 mM Tris-HCl (pH 9.0) containing 50% v/v MeOH.
2. 25 mM Tris-HCl, 1 mM EDTA (pH 8.0).
3. Stock enzyme (LysC): as for **Subheading 2.3.**, **item 3**.
4. 30% v/v MeCN containing 2.5% v/v aqueous TFA.
5. 60% v/v MeCN containing 2.5% v/v aqueous TFA.

2.5. Enzymatic Digestion on Immobilon-P

1. 70% v/v Aqueous MeCN.
2. 25 mM NH$_4$CO$_3$, 1% octyl-β-glucoside, 10% v/v MeOH (digest buffer).
3. Stock enzyme (LysC): as for **Subheading 2.3.**, **item 3**.
4. 50% v/v Ethanol/50% v/v formic acid (HCOOH) (98%) (prepared immediately prior to use).
5. 10% v/v Aqueous MeCN.

2.6. Microcolumn Chromatography

1. μ-Guard™ column, 300 μm id × 1 mm, C8 packing, 300-Å wide-pore (LC packings, San Francisco, CA) (*see* **Note 1**).
2. Lite-Touch® ferrules for $\frac{1}{16}$-in. od tubing.
3. Inlet tubing, 2 cm, 300 μm id, $\frac{1}{16}$-in. od Teflon® tubing (PE-Applied Biosystems Division, Foster City, CA) or similar.
4. Outlet tubing, 2 cm, 0.005-in. id, $\frac{1}{16}$-in. od PEEK (Red) (Upchurch Scientific, Oak Harbor, WA).
5. Hamilton syringe, 10 μL with a fixed or removeable beveled needle and Chaney adapter (Baxter Healthcare, Irvine, CA).
6. 1% v/v Aqueous HCOOH (98%) (make fresh weekly).
7. 10% v/v Increments, or as desired, of MeCN to 90% in 1% v/v aqueous HCOOH (98%) (make fresh weekly).
8. MeOH.
9. Parafilm™.

2.7. MALDI-MS Reagents

1. α-Cyano-4-hydroxycinnamic acid (4HCCA, 97%, Aldrich, St. Louis, MO) 10 g/L (50 mg/5 mL) in 70% v/v MeCN containing 30% 0.1% v/v aqueous TFA.
2. 33 mM α-cyano-4-hydroxycinnamic acid in MeCN:MeOH:dH$_2$O (5:3:2 v/v) (Hewlett-Packard, Palo Alto, CA).
3. 29 mM α-cynao-4-hydroxycinnamic acid in HCOOH:dH$_2$O:2-propanol (1:3:2 v/v) also referred to as formic acid:water:isopropanol, FWI; *see* **item 4**).
4. HCOOH:dH$_2$O:2-propanol (1:3:2 v/v) (FWI).
5. 10 μM bovine insulin β-chain, oxidized (Sigma).
6. Ice-cold 0.1% aqueous TFA.
7. Synthetic peptide Pro$_{14}$Arg.
8. Thionyl chloride (99%+, Aldrich). NB: Only use this reagent in a well-ventilated fume hood, since it reacts violently with water to yield HCl vapor.
9. Methanol, anhydrous (99%+, Aldrich).
10. Heating block capable of maintaining 50°C.

3. Methods
3.1. Generation of Peptide Fragments
3.1.1. In-Gel Digestion Protocol

This protocol is for proteins visualized in an SDS-PAGE gel using the "reverse-staining" protocol of Ortiz et al. *(9)*. Briefly, this method involves

immersion of the gel in 0.2 *M* Imidazole for at least 15 min after which time the solution is changed to 50 m*M* ZnCl$_2$ until the background becomes opaque. The gel is then rinsed and stored in dH$_2$O at 4°C. The stain is sensitive to ~100 fmol (loaded on the gel) level, except for heavily glycosylated and sialylated proteins, which are not readily observed *(10)*. The following protocol was originally described for use with Coomassie blue-stained proteins *(2)*. We have not included a reduction and alkylation protocol, but this can be used if required (*see* Chapter 52). The only difference between that method and what is listed below is the use of citric acid for "destaining" or mobilizing the proteins.

1. Clean work area, microfuge tubes, and all utensils that will be used with 50% v/v MeOH/0.1% v/v TFA solution, and let dry (*see* **Note 2**).
2. Identify the band or spot of interest on the gel, and carefully excise (*see* **Note 3**, and **Fig. 1** for schematic). Do not touch the gel except with forceps. Remove the gel piece to a "cleaned" area where the piece (depending on its size) can be chopped into ~1 mm^2 or smaller cubes for digestion. Excise a blank region (containing no protein) from the same gel that is equivalent in size to the band or spot of interest to serve as a control (*see* **Note 4**).
3. Place the chopped pieces into a microfuge tube, and wash with a 50-m*M* citric acid solution (usually 200 μL, but depending on band size, more may be used without effect) for 20 min on a rotating mixer. This mobilizes the proteins in the reverse-stained gel. Decant the citric acid solution when completed.
4. Add 500 μL 20 m*M* NH$_4$HCO$_3$/50% v/v MeCN to the tube containing the gel pieces and again place on the rotating mixer. After 30 min, replace the wash solution with a fresh buffer, and continue to wash for another 30 min. Decant the wash solution when completed.
5. Place the tubes in a vacuum concentrator (e.g., Speed Vac, Savant), and dry the gel pieces completely (minimum of 30 min) (*see* **Note 5**).
6. To the dry pieces in the bottom of the tube, add 1 μL of stock enzyme (LysC at 0.1 μg/μL in dH$_2$O) and 15 μL of 20 m*M* NH$_4$HCO$_3$, 1.0 m*M* CaCl$_2$. Leave at room temperature for 15 min. Repeat this step until the gel pieces are totally rehydrated. Add 3× more digest buffer than what was used for the rehydration (i.e., if two additions of enzyme and digest buffer were added, in this step, add an additional 90 μL of digestion buffer) (*see* **Note 6**). Gently mix the tube and place in a 37°C water bath for overnight digestion.
7. After brief centrifugation, remove the digest buffer supernatant, add it to a "clean" microfuge tube, and reduce the volume of the supernatant to ~5 μL by vacuum concentration in a Speed Vac. Make every effort not to let the samples dry completely to avoid additional loss of peptides to the tube. Store these tubes for later microcolumn clean-up (*see* **Subheading 3.2.**). To the gel pieces now add 200 μL of 1% v/v TFA and sonicate in a warm (~37°C) sonication bath for 30 min (*see* **Note 7**).
8. Again, after a brief centrifugation, remove the buffer, place it in a "clean" microfuge tube, and reduce the volume to 1–2 μL without drying (if possible).
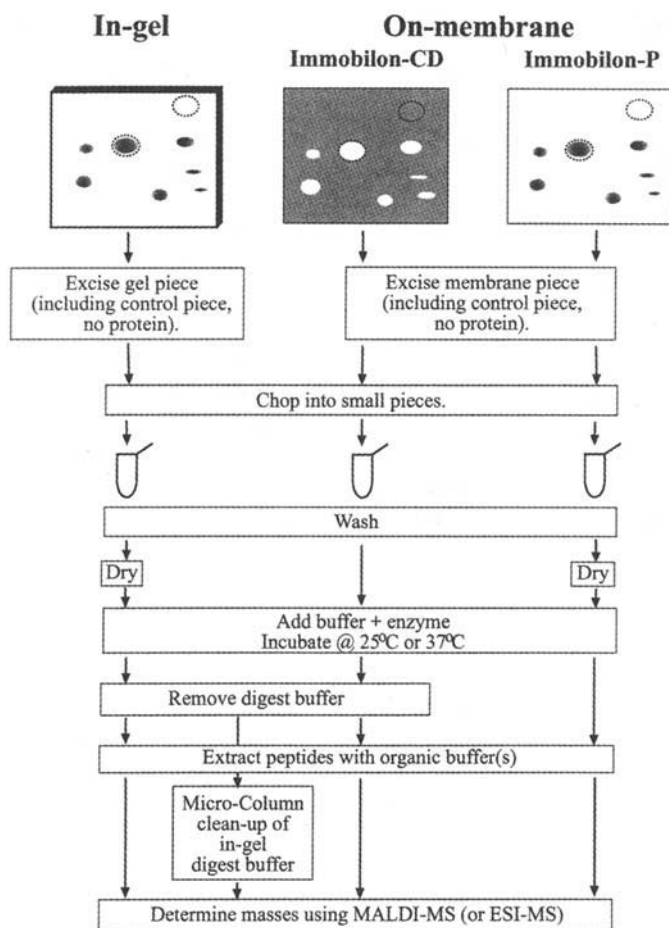
Fig. 1. Flowchart outlining methods for obtaining peptides from gel-separated proteins by either digestion in-gel or on-membrane for subsequent MS analysis. In all cases, the spot (or band) of interest, together with a control piece containing no protein, is excised and subjected to digestion and extraction of the peptides. For the Immobilon-CD protocol, the washing and chopping into small pieces steps can be reversed.

These samples are now ready for MALDI-MS analysis (*see* **Notes 8** and **9**). Next, add 200 μL of 60% v/v MeCN/0.1% v/v TFA to the microfuge tube containing the gel pieces and again place in a warm (~37°C) sonication bath for 30 min (*see* **Note 7**).

9. After a brief centrifugation, remove the buffer, place it in a clean microfuge tube, and reduce the volume to 1–2 μL without drying completely (if possible). These samples are now ready for MALDI-MS analysis (*see* **Note 10**).

3.1.2. Immobilon-CD Digestion Protocol

Proteins are generally visualized on Immobilon-CD using a commercial negative stain kit (Immobilon-CD Stain Kit, Millipore, Beverly, MA) that yields a purple background with white areas indicating the presence of protein. The sensitivity of the stain is about 0.5 pmol of protein loaded on the gel *(10)*.

1. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/ 0.1% v/v TFA solution, and let dry.
2. Identify the band or spot of interest on the wet membrane and carefully excise (*see* **Note 11** and **Fig. 1** for schematic). Do not touch the membrane. Place the membrane pieces into separate microfuge tubes, and wash in 200 μL of 20 m*M* Tris-HCl (pH 9.0), 50% MeOH four times prior to any further manipulation to remove any residual SDS from the membrane.
3. Remove the membrane piece to a cleaned area where the piece (depending on its size) can be cut into ~1 mm^2 or smaller squares for digestion. Excise a piece of blank region (containing no protein) from the same membrane of equivalent size as the band or spot of interest to serve as a control (*see* **Note 4**). Keep the membrane pieces wet while cutting them with a drop of dH$_2$O if necessary.
4. Add the diced membrane pieces to a new 500-μL microfuge tube and add 2 μL (for a membrane piece of ~6 mm^2) or 10 μL (for a membrane piece of ~20 mm^2) of 25 m*M* Tris-HCl, 1 m*M* EDTA (pH 8.0). Then add either 0.4 or 2 μL of Endo Lys-C (5 μg vial reconstituted in 50 μL deionized water) for the 6- or 20-mm^2 membrane pieces, respectively. Incubate at 25°C for at least 20 h (*see* **Note 12**).
5. Remove the digest solution containing any passively eluted peptides and store in a microfuge tube. Extract the membrane pieces with 3 or 10 μL (6- or 20-mm^2 membrane pieces, respectively) of 30% MeCN/2.5% TFA. After vortexing for at least 30 s, centrifuge the samples, remove the extract, and store separately in another microfuge tube. Repeat the procedure using the same volume of 60% MeCN/2.5% TFA with sonication for 3 min. The three aliquots are now ready for MALDI-MS analysis, preferably on the same day (*see* **Note 8**).

3.1.3. Immobilon-P Digestion Protocol

The proteins can be visualized on Immobilon-P using Coomassie blue or Sulforhodamine B as described *(4)*. The sensitivity of Coomassie is about the same as that for Immobilon-CD, whereas Sulforhodamine B can visualize proteins to the ~100-fmol level.

1. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/0.1% v/v TFA solution, and let dry.
2. Identify the band or spot of interest on the dry membrane, and carefully excise (*see* **Note 10** and **Fig. 1** for schematic). Do not touch the membrane. Remove the sample to a cleaned area where the piece (depending on its size) can be cut into ~1-mm^2 or smaller squares for digestion. Excise a piece of blank membrane (containing no protein) the same size as the band or spot of interest to serve as a

control (*see* **Note 4**). Rehydration of the membrane pieces while cutting them with a drop of 50% v/v MeOH/dH$_2$O is sometimes helpful.

3. The Coomassie blue-stained membrane pieces are then put into a microfuge tube and washed/destained using 200 μL of 70% v/v MeCN and vortexing. When the bands are totally destained (~30 s), pipet off the solvent and dry the pieces in a vacuum concentrator for 10 min or until completely dry.

4. To the dry membrane pieces, add 1 μL of your stock enzyme (LysC at 0.1 μg/μL in dH$_2$O) and just enough digest buffer (NH$_4$CO$_3$/octyl-β-glucoside/MeOH) to cover all of the membrane pieces, usually 3–10 μL, depending on the size of the protein band. It is sometimes helpful at this point to use a clean gel loading pipet tip or needle of a syringe to keep the dry membrane pieces in the digest buffer while they rehydrate (they tend to float on the surface of the liquid in the tube). Incubate the sample at 26–27°C overnight.

5. Remove and store the digest buffer separately from the tube (*see* **Note 13**). If necessary, this can be cleaned up later using the microcolumn (*see* **Subheading 3.2.** and **Note 14**). Add 10 μL of freshly prepared 50% v/v ethanol/50% v/v HCOOH, and place in a sonicator for 30 min. After sonication, remove the extract, and dry completely in a vacuum concentrator. Rehydrate in 1–5 μL of 10% v/v MeCN/1% v/v HCOOH and perform MALDI-MS analysis.

3.2. Microcolumn Chromatography for Sample Clean-Up

This technique has been developed as a manual chromatographic clean-up step to remove salts and contaminants from samples (digest supernatants), which would be below the detectable limit for normal microbore chromatography (i.e., a few pmol or less loaded on the gel). This quick, easy clean-up step has not only helped the overall recovery of peptides from techniques of this nature, but has also dramatically lowered the effective limit of in-gel and on-membrane digests to low-picomolar to subpicomolar levels *(7–12)*.

1. Assemble the microcolumn by attaching the outlet and inlet tubing to the μ-Guard column using the Lite-Touch ferrules and tighten by hand. The outlet tubing (Red PEEK) is shaved to yield a conical end allowing 3-μL droplets to form. This will not occur with a blunt end.

2. Equilibrate the microcolumn using 30 μL (3 × 10 μL) MeOH using a 10-μL Hamilton syringe to introduce the solution into the inlet tubing (*see* **Note 15**).

3. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/ 0.1% v/v TFA solution, and let dry.

4. Prepare the digest supernatant for microcolumn clean-up by reducing its volume to 3–10 μL.

5. Equilibrate the column with 30 μL of 1% v/v HCOOH at a flow rate of approx 1–2 μL/s.

6. Using a 10-μL Hamilton syringe, load your sample into the syringe, and attach to the column inlet. Using the same approximate flow rate as with the equilibration, pass the sample over the column, collecting the flowthrough as the "void/wash"

into a microfuge tube. Reload 10 μL of 1% v/v HCOOH back into the same syringe and pass over column. Continue to collect this wash into the void/wash tube (*see* **Note 16**).

7. Rinse the syringe quickly (2–3×) with 30% v/v MeCN/1% v/v HCOOH (*see* **Note 17**). Load 3 μL of 30% v/v MeCN/1% v/v HCOOH into the syringe, and attach to the column inlet. Again at the same approximate flow rate, pass the solvent over the column, collecting the eluant in a microfuge tube labeled 30% MeCN.

8. Repeat **step 7** using 3 μL of 70% v/v MeCN/1% v/v HCOOH, and elute into an appropriately labeled tube.

9. Repeat **step 7** using 6 μL of 90% v/v MeCN/1% v/v HCOOH, and elute into an appropriately labeled tube. The larger elution volume is used to attempt to ensure complete recovery of peptides from the column.

10. Wash the column with 30 μL of 90% v/v MeCN/1% v/v HCOOH.

11. Repeat the series of steps on the next sample.

12. When all samples are finished, clean the column in MeOH (at least 3 × 10 μL) and wrap the column in Parafilm, being sure the inlet and outlet are sealed to avoid drying out.

13. Proceed with MALDI-MS analysis on all fractions collected (*see* **Note 18**).

3.3. MALDI-MS

3.3.1. Matrix Preparation and Selection

1. Matrix solutions are prepared in small volumes (5 mL or less) so that they will not be stored for excessive periods of time. Add the appropriate quantity of powdered 4HCCA (listed in **Subheading 2.7.**) to the organic solution, and then add the remaining solution(s). The matrix solution is stored in a container (e.g., glass vial or microfuge tube) protected from the light and may need to be centrifuged briefly to pellet any undissolved chemical prior to use (*see* **Note 19**).

2. The matrix 4HCCA listed in **Subheading 2.7.** can be dissolved in different solvents. In general, we use the commercial matrix preparation, but this is for convenience only and we have not found an appreciable difference in spectra obtained with this and the MeCN/TFA solvent mixture, although one should remember that the commercial matrix preparation also has a limited life. However, the FWI mixture (**Subheading 2.7.**, **item 3**) can dramatically change the peptides observed in complex mixtures *(13)*, and this can be particularly useful when both the "standard" and FWI-based matrices are used in parallel, since more peptides can be observed than with either matrix alone.

3. 4HCCA is the most widely used matrix for peptide mixtures (which this chapter is concentrating on), and can also be employed for proteins up to the size of at least serum albumin. However, sinapinic acid is often used to analyze proteins (*see* **Note 20**).

3.3.2. Preparation and Loading of the MALDI-MS Target

1. Rinse the MALDI-MS target with MeOH, and wipe dry with a lint-free tissue, or follow the procedure described by the MALDI-MS manufacturer.

2. The simplest MALDI-MS sample preparation method is to add a small aliquot of the sample ~0.3 µL (in the low to subpmol range) to the target well on the sample slide followed by an equal amount of matrix. This solution is mixed in the pipet tip, and then allowed to dry at room temperature. When small volumes are used, drying only takes a few minutes.

3. Other sample preparation methods include mixing an aliquot of the sample and the matrix in a separate microfuge tube prior to loading the mixture on the target well. The sample/matrix mixture can also be subjected to vacuum to assist in even crystallization.

4. After the sample has dried, and hopefully an even crystalline surface is visible, the sample slide is ready for loading into the MALDI-MS instrument and data analysis.

3.3.3. Calibration

It is important to ensure that masses measured with the MALDI-MS are as accurate as possible. This can be achieved through "external" calibration where the calibrant is applied to a target well separate from the sample, or by "internal" calibration where the calibrant is mixed with the sample. In either case, the aim is to use ions of known mass, which bracket the sample to be measured. We recommend incorporating a calibrant with every sample set to provide the opportunity for external calibration with each experiment, or at least to confirm that the instrument calibration is stable.

1. Calibration routines are instrument- (software) dependent, but in their simplest form, they employ a two-point calibration using a matrix ion and a large peptide of known molecular mass.

2. Calibration for peptide mixtures can be performed using 0.3 µL of a 10 µ*M* solution of the oxidized B chain of bovine insulin (MH$^+$ 3496.9) dissolved in dH$_2$O into the target well, followed by 0.3 µL of matrix. A spectrum averaged from at least 50 shots is obtained at a laser fluence just above threshold to ionize the calibrant, in the same manner that sample data is acquired. The signal from the calibrant (oxidized B-chain of bovine insulin), together with either the dehydrated matrix ion of 4HCCA at MH$^+$ 172.2 or the matrix dimer at MH$^+$ 379.2, allows us to bracket many of the peptides generated in a LysC or tryptic digest.

3. The specific software calibration routine is then followed to fit a straight line to these two ions (matrix and calibrant).

4. Internal calibration can be performed after the sample spectrum has been obtained by redissolving the sample in a small volume (~0.2 µL) of calibrant mixed with matrix (e.g., a ratio of calibrant:matrix of 4:1). The amount of calibrant applied needs to yield an intensity equivalent to that of the sample. This can be judged from the intensity of the calibrant in the external calibrant, although ionization suppression can sometimes occur (*see* **Note 21**).

3.3.4. On Probe Sample Clean-up

Occasionally, despite the procedures outlined above, some samples may still not yield signals by MALDI-MS. There are a number of on-probe sample clean-

up procedures, but it is not the aim of this chapter to detail them all. The following procedure first described by Beavis and Chait *(14)* is one of the simplest, and is often very effective. It relies on the difference in solubility between the peptide/protein-matrix crystals, and salts and other low-mol-wt contaminants. The salts are soluble in ice-cold acidic solution, whereas the peptide/protein-matrix crystals are not.

1. Add 2–3 μL of ice-cold 0.1% TFA to the dried sample in the target well for ~5 s, and then remove this with the same pipet (or blowing it off with forced air).
2. This can be repeated at least twice (*see* **Note 22**).

3.4. Peptide-Mass Searches

After the accurate peptide masses are obtained from the protein band or spot, they can be used in search programs to determine whether the protein exists in the current full-length protein sequence databases, or translations of nucleotide sequence databases. There are a number of publicly accessible sites on the internet (World Wide Web), which can be used to search these databases. The following is a list of servers currently available together with their affiliations and URL addresses. All supply full instructions on-line concerning to their use.

1. MassSearch from CBRG, ETHZ, Zurich: http://cbrg.inf.ethz.ch/subsection3_1_3.html.
2. MOWSE search program from SEQNET, Daresbury: http://www.dl.ac.uk/SEQNET/mowse.html.
3. MS-Fit (and MS-Tag) from the University of California, San Francisco: http://rafael.ucsf.edu/MS-Fit.html.
4. Peptide Mass Search from Max Delbruck Center for Molecular Medicine, Berlin: http://www.mdc-berlin.de/~emu/peptide_mass.html.
5. PeptideSearch from EMBL, Heidelberg: http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html.
6. ProFound (and PepFrag) search program from Rockefeller University, New York: http://chait-sgi.rockefeller.edu/cgi-bin/prot-id/1/1.

It is essential to obtain the most accurate masses possible. Therefore, we recommend internal calibration for such analyses as described above (i.e., we obtain externally calibrated spectra prior to adding a small amount of calibrant to the sample).

The various peptide–mass search programs each have their own idiosynchrasies, but all require a set of peptide masses (together with a stated tolerance or mass accuracy), the enzyme or chemical reagent used to generate the peptides, whether the cysteine residues have been modified (i.e., carboxylmethylated, and so forth), and whether missed cleavage sites should be considered. Additional input to the program can include modifications to other specified amino acid residues (e.g., methionine sulfoxide), peptide masses

following deuteration (an amino acid composition-dependent mass increase), a selection of which database(s) to search, whether the search should be restricted to a subset of proteins whose intact mass falls within a specified range around the mass of the unknown protein of interest, and the species from which the sample was derived (for reviews, *see* **refs. *1*, *15*, and *16***). The Daresbury program can use further information on individual peptides, such as partial composition and partial sequence. It should be noted that electrophoresis can induce artifactual modifications, e.g., acrylamide adducted to free cysteines and the *N*-terminus, and methionine oxidation (*see* **Note 23**).

Most programs will yield some result given a set of input peptide masses even if only a few masses are found to match. Rarely do all of the input peptide masses match with the top-ranked candidate. Therefore, it is critical to attempt to determine how these peptide masses arose. The following is a list of possibilities for peptide masses that are not matched with the top-ranked candidate:

1. The correct protein was identified and the nonmatching peptides are owing to either posttranslational modification (including artifactual) or processing (*see* **Note 24**).
2. The correct protein was matched, but some peptides were derived following either unspecific cleavage of the protease or specific cleavage from a contaminating protease (*see* **Note 25**).
3. The correct protein was identified, but was part of a mixture of two or more proteins (*see* **Note 26**).
4. A homolog (or processing/splice variant) from the same or a different species was identified (*see* **Note 27**).
5. The result is a false positive (*see* **Note 28**). The possibility should not be overlooked that the real protein does not exist as an entry in the database being searched and may be truly novel.

When possible, it is advisable to attempt to gain further information on the peptides by either chemical treatments or chemical or mass spectrometric sequencing (*see* **Subheading 3.6.**). Of course this is dependent on the quantity of peptide available for further analysis.

3.5. Postsource Decay MALDI-MS

To improve the confidence in the results of a peptide mass search, one needs to determine additional primary structural information on the peptides observed *(1)*. One means of achieving this aim is to isolate the peptide in the gas phase of the mass spectrometer and induce fragmentation of the peptide and measure the mass of the fragment (or daughter) ions. There are a number of mass spectrometric instrument designs with various ionization sources that allow gas-phase isolated peptides to be fragmented, e.g., a triple-quadrupole MS, an ion-trap MS, and a time-of-flight (MALDI-MS) when the instrument is fitted with either

a variable voltage reflector or curved-field reflector. We will describe the use of the latter type of MALDI-MS instrument, i.e., a curved-field reflector instrument. However, it should be noted that the same principles apply to the use of a MALDI-MS fitted with a variable voltage reflector. Fragment-ion spectra observed in a MALDI-MS instrument are generated by "postsource decay" or metastable fragmentation of the ions during the ionization process (*see* **Note 29**). This is distinct from "in-source" or "prompt" fragmentation that occurs very early during the ionization (and therefore acceleration) process, and so the fragment ions are resolved in the linear dimension because of their different velocities (e.g., peptides linked by a single disulfide bond have been observed to undergo prompt fragmentation *[17]*). Fragmentation is sequence-specific, and generates the same ion series observed during other types of gas-phase fragmentation, including both single- and multiple-bond cleavages (*see* **Note 30**). It should be noted that if the peptide carries a posttranslational modification, the bond linking it to the peptide may be weaker than the peptide bonds themselves, so the fragment ions observed sometimes result from loss of the modifying group and little if no peptide bond fragmentation (e.g., O-linked carbohydrate and, sometimes, phosphate on serine or threonine residues).

3.5.1. Matrix Selection, Sample Loading, and Calibration

1. The most commonly used matrix for PSD-MALDI-MS is 4HCCA, since this is considered a relatively "hot" matrix, i.e., there is often considerable metastable fragmentation. The two other most common matrices, sinapinic acid (3,5 dimethoxy cinnamic acid) and gentisic acid (or dihydroxy benzoic acid), yield less metastable decay products. Matrix preparation and sample loading are as described in **Subheadings 3.3.1.** and **3.3.2.**

2. PSD-MALDI mass spectra can only be calibrated externally, since the sample is a single molecular species. The manufacturer will have a specific protocol to follow, but we use the calibrant first described by Cordero et al. *(18)*, which is a synthetic peptide of 14 prolines with an arginine C-terminus $(Pro_{14}Arg)(M_{ave}H^+ = 1534.8)$. However, other peptides, such as Angiotensin II, whose fragment-ion spectrum is well known, can also be employed for fragment-ion calibration. The $Pro_{14}Arg$ peptide readily generates strong *y* series ions with a few *b* series ions as well as the proline immonium ion, thereby providing an excellent range of fragment-ions for calibration (*see* **Fig. 2**).

3. Using the Kompact MALDI IV, the reflector is first calibrated with intact molecular ions in the same manner as in linear mode (*see* **Subheading 3.2.**, **step 3**), and then the calibrant peptide is fragmented, and the masses of the fragment-ions observed without any correction are entered into the calibration program. The expected fragment-ion mass for each selected ion is then entered, and the software uses a curve-fitting program to construct a calibration curve for use with other spectra.

4. Although we routinely check the calibration, in our experience, the calibration is very stable. This is expected, since it is an instrument-dependent parameter. The
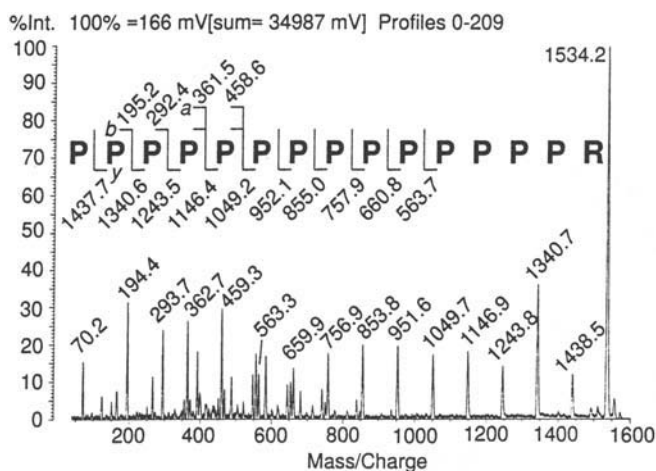
Fig. 2. PSD-MALDI-mass spectrum of $Pro_{14}Arg$ ($M_{ave}H^+ = 1534.8$) using a curved-field reflector MALDI-MS. The observed fragment-ion masses following calibration are listed over the peaks used in the calibration routine. The expected fragment-ion masses for the observed *a*, *b*, and *y* series ions are listed above and below the peptide sequence. The immonium ion of Pro has a mass of 70.1 u. The spectrum was smoothed over four bins, and a baseline subtraction of 40 was used.

same would be expected for MALDI-MS instruments where the reflector is scanned with different set voltages as long as these are stable between analyses.

3.5.2. Methyl Esterification
as a Means to Assist Fragment-Ion Interpretation

Pappin et al. *(3)* described a simple method for methyl esterification of peptides that can be used both for peptide-mass mapping in linear MALDI-MS and PSD-MALDI-MS (*see* **Note 31**). This method, in most instances, quantitatively esterifies all carboxylic acid groups on peptides to the corresponding methyl esters, thereby increasing the mass by 14 u for each group. A free C-terminus will result in a mass increase of 14 u, and any acidic residues (Asp or Glu) will result in additional 14-u mass increases for the peptide, allowing the number of acidic residues to be calculated. This is of assistance to both peptide-mass and fragment-ion searches as well as for *de novo* interpretation of the fragment-ion spectrum. A simple example using a four-residue peptide of sequence FGSR is shown in **Fig. 3A** and following methyl esterification in **Fig. 3B**.

1. Aliquot a portion, 1–1.5 μL, of the sample (either digest or individual peptide fraction) and dry by vacuum concentration in a 500-μL microfuge tube (e.g., in a SpeedVac) (*see* **Note 32**).

A

%Int. 100% =293 mV[sum= 36680 mV] Profiles 0-124

121.1 ... 466.5

117.2 *175.4
158.6 205.2
245.1 *302.4
113.2
*262.0 *319.7
43.4 60.9 71.2 88.3 131.0 145.8 177.3 292.4

Mass/Charge

B

%Int. 100% =196 mV[sum= 35393 mV] Profiles 0-179

121.3 480.9

172.6
177.6
205.5 259.3
316.6
276.1 334.0
131.2
189.4
43.2 61.0 71.3 88.3 113.7 146.1

Mass/Charge

C

a 177.2 MaveH⁺ = 466.5
b 205.2 292.3

F | G | S | R

319.3 262.3 175.2 y
302.3 245.3 158.2 y -17

Internal Fragments
GS-28= 117.1
GS = 145.1

Immonium Ion Masses
Phe = 120.2
Ser = 60.1
Arg = 129, 112, 87, 70, 43

Fig. 3. PSD-MALDI-mass spectrum, using a curved-field reflector MALDI-MS, of a peptide of sequence FGSR with and without methyl esterification. Panel (**A**) shows the fragment-ion spectrum without methyl esterification, panel (**B**) with methyl esterification, and panel (**C**) shows the theoretical fragment-ion masses for the peptide FGSR (corresponding to those labeled in panels A and B). The y and y-17 (loss of ammonia) series ions are labeled with an * in panel A. All of the ions (y and y-17 series ions, and the intact molecule) that increase in mass by 14 u following methyl esterification have their observed masses boxed in panel B. Both spectra were smoothed over two bins with no baseline subtraction.

2. Make a 1% v/v thionyl chloride solution in anhydrous methanol (*see* **Note 33**).
3. Add 3–6 µL of the thionyl chloride solution (3× the volume of the original sample). Cap the tube, and heat at 50°C for 30 min in a heat block (*see* **Note 34**).
4. Dry the reaction mixture by vacuum concentration, and resuspend with 3 µL of 30% v/v MeCN/1% v/v HCOOH (**Subheading 2.6.**, **item 7**).
5. An aliquot of this solution can be applied to the MALDI sample well, and allowed to dry prior to adding matrix (*see* **Subheading 3.3.2.**).

3.5.3. Interpretation of PSD-MALDI-MS Spectra

We do not usually manually interpret PSD-MALDI-MS spectra, instead we use the uninterpreted fragment ions in a computer search (*see* **Subheading 3.6.**). If all of the ions used in the search have not been matched by the program, we then use the matched sequence to see if any unmatched ions can be explained by fragmentations not included in the search program (e.g., some of the search programs do not include internal fragmentations). However, it is certainly possible to interpret manually some PSD-MALDI-MS spectra. The simple spectrum in **Fig. 3** is a good example. The following is a description of how the spectrum could be interpreted, in a manner similar to that described by Kaufmann et al. *(19)*. The calculated fragment ions for this peptide are shown in **Fig. 3C**. Basically, the strategy is to look for immonium ions and then determining whether an ion series can be identified by mass differences (some of which could correspond to the identified immonium ions). Once a sequence has been formulated, one attempts to correlate all of the observed ions in the spectrum with the sequence.

1. The immonium ion region can often provide information on the amino acid content of the peptide (*see* **Table 1** for immonium ion masses). In **Fig. 3A**, the following ions are observed in this region: 43, 61, 71, 88, 113, 117, 121, 131, 145, and 158. Given the expected mass tolerance (±1 u), these ions could represent immonium ions from Arg (expected 43, 70, 87, 112, and 129), Pro (expected 70), Ser (expected 60), Leu/Ile (expected 86), Phe (expected 120), and Trp (expected 159). However, given the ion intensities—Pro and Leu/Ile too weak, and Trp too strong—these are not likely candidates. The 117 and 145 ions are not matched.
2. The peptide was derived from a tryptic digest, so it would be expected that the C-terminus would be Lys or Arg (although of course there are exceptions, e.g., the C-terminal peptide or a nonspecific cleavage). A strong ion at 175.4 is observed in **Fig. 3A**, which is the expected y_1 ion for an Arg C-terminus (expected 175.2). The neutral loss ion at 158.6, which is expected for an Arg, is also present (given the intensity, a more plausible explanation than a Trp immonium ion). If Lys were at the C-terminus, the y_1 ion would be 147.
3. We can now look for ions with masses between 232 (175 + 57, Gly) and 361 (175 + 186, Trp), because the next series ion has to have a mass between that of an additional Gly or Trp residue (if no residues are modified). A number of ions are present in this mass range. Since there is an Arg at the C-terminus, the *y*-series

Table 1
Residue Masses of Amino Acids Together
with Their Corresponding Immonium Ion Masses[a]

Amino acid	Abbreviations— three letter, single letter	Residue mass[b]	Immonium ion mass[b]
Glycine	Gly (G)	57	30
Alanine	Ala (A)	71	44
Serine	Ser (S)	87	60
Proline	Pro (P)	97	70
Valine	Val (V)	99	72
Threonine	Thr (T)	101	74
Cysteine	Cys (C)	103	76
Isoleucine	Ile (I)	113	86
Leucine	Leu (L)	113	86
Asparagine	Asn (N)	114	87
Aspartate	Asp (D)	115	88
Glutamine	Gln (Q)	128	101
Lysine	Lys (K)	128	129, 101, 84[c]
Glutamate	Glu (E)	129	102
Methionine	Met (M)	131	104
Histidine	His (H)	137	110
Phenylalanine	Phe (F)	147	120
Arginine	Arg (R)	156	129, 112, 100, 87, 70, 43[c]
Tyrosine	Try (Y)	163	136
Tryptophan	Trp (W)	186	159

[a]The values were obtained from Jardine *(20)*, and Spengler et al. *(21)*.
[b]All masses are given as average integer values.
[c]Arginine and lysine both exhibit multiple immonium ions, and these are listed (they are not of equal intensity).

ions are expected to exhibit strong neutral losses. The ions at 245.1 and 262.0 could represent a *y* and *y*-17 pair, as could 302.4 and 319.7. In fact, the mass difference between 262.0 and 175.4 is 86.6 (which could correspond to Ser, observed in the immonium ions). The difference between 319.7 and 175.4 (144.3) does not match an amino acid residue. However, the difference between 319.7 and 262.0 of 57.7 could correspond to Gly. This would make the sequence RSG, reading from the C-terminus.

4. The difference in mass from the molecular ion (466.5) and the last assigned *y* series ion (319.7) is 146.8, which matches with Phe (expected mass of 147.2). Thus, the complete *y* series has been observed, and given the immonium ion masses indicated—Arg, Ser, and Phe—the sequence can be assigned with some confidence as FGSR.

N-Terminal Ions
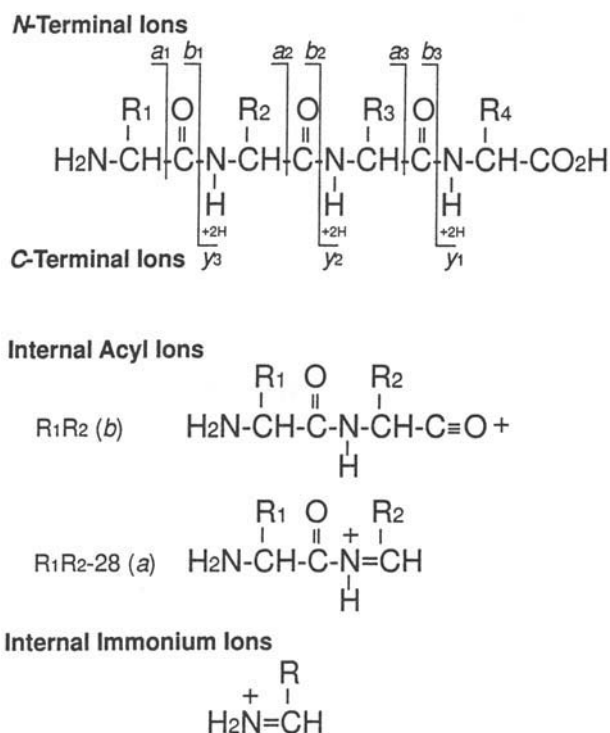
Fig. 4. Fragmentation nomenclature for the most common positive ions observed by PSD-MALDI-MS (after *22,23*). Fragmentation is usually only unimolecular, i.e., only one break occurs in the peptide backbone, and the charge is retained on either the N-terminus (*a* and *b* series ions) or the C-terminus (*y* series ions). However, internal acyl ions and immonium ions can also be formed from multiple fragmentation events. The internal acyl ions are referred to by their amino acid sequence (e.g., R_1R_2) (in a form similar to *b* series ions), or their sequence –28 (for those similar to *a* series ions). There are also neutral losses from the internal acyl ions where part of an amino acid side chain is lost (e.g., ammonia [17 u] can be lost from Q, K, and R side chains, and water [18 u] can be lost from S and T side chains). Similar losses can also occur from the molecular ion (a very good list can be found at the MS-Tag WWW site, http://rafael.ucsf.edu/MS-Fit.html). Amino acid side chains are referred to as R_1, R_2, and so on, in the formulae.

5. One can also examine the spectrum for ion pairs with a mass difference of 28, which could represent *a* and *b* series ions or internal fragments (which can also exit as a pair of ions separated by 28 [*see* **Fig. 4**]). There are two sets of ions that fit this criteria: 117.2–145.8, and 177.3–205.2. Given our interpretation of the sequence from the *y* series ions, these ion pairs correspond to the internal fragment GS and GS-28, and a_2 and b_2, respectively. The b_3 ion is also observed at

292.4. The mass difference between the molecular ion and the b_3 ion corresponds to 156.1 + 18, which matches Arg + water. We could also have used dipeptide tables (such as those listed at the WWW site of Burlingame's group at http://rafael.ucsf.edu/MS-Fit.html), which in this case reveal that GS is the only pair with a mass close to that observed, whereas the 205 could be from CT, M(Ox)G, or the correct pair, FG. Therefore, we have been able to assign ions consistent with the putative sequence FGSR using the strategy of observing the immonium ion masses followed by looking for mass differences that correspond to amino acid residues (and pairs of ions separated by either a neutral loss of ammonia [17 u] or water [18 u] or an *a-b* ion series pair [28 u]), in this case, starting with a putative y_1 ion.

6. The methyl esterification experiment shown in **Fig. 3B** provides additional confirmation of the sequence interpreted above. The mass of the parent ion only increased from 466.5 to 480.9 corresponding to one methyl group, which would be expected to be added to the C-terminal carboxyl residue. Therefore, all of the *y* series ions would be expected to shift by 14 u, and no other ions should shift compared to unmodified spectrum in **Fig. 3A**.

7. As expected, all of the putative *y*-series fragment-ions have increased in mass by 14 u (boxed in **Fig. 3B**), confirming their identity. None of the other fragment-ions have shifted. When methyl esterification is possible, even if acidic residues are present in the peptide, and therefore increase the parent-ion mass by multiples of 14 u, careful examination of the spectrum can often allow almost complete interpretation of spectrum. An example of when this becomes difficult is when there is one acidic residue and it is at the N-terminus. Then all fragment-ions (except internal fragment ions) are shifted by 14 u, and the parent-ion mass is 28 u higher than the unmodified ion. The immonium ion masses of Glu and Asp are also increased by 14 u following methyl esterification.

3.6. Fragment Ion Searches

Search programs, in addition to the peptide-mass search programs (*see* **Subheading 3.4.**), which use either partially or uninterpreted fragment-ion spectra to search protein or translated nucleotide sequence databases, are publicly available on the internet (WWW). The following is a list of servers currently available together with their affiliations and URL addresses. All supply full instructions on-line concerning their use.

1. MOWSE search program from SEQNET, Daresbury: http://www.dl.ac.uk/SEQNET/mowse.html.
2. MS-Tag from the University of California, San Francisco: http://rafael.ucsf.edu/MS-Fit.html.
3. ProFound (and PepFrag) search program from Rockefeller University, New York: http://chait-sgi.rockefeller.edu/cgi-bin/prot-id/1/1.
4. PeptideSearch from EMBL, Heidelberg: http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html.

The uninterpreted fragment-ion programs from San Francisco (MS-Tag) and New York (PepFrag) require input of the fragment-ion masses together with the ion series from which they may have been derived (e.g., *a*, *b*, *y*, neutral losses, and so forth). The Daresbury program is not really a fragment-ion search program, but it does allow information to be added to individual peptides in a peptide-mass search, such as how many acidic residues are present (from a methyl esterification experiment) or what amino acid residues are present (which can be derived from immonium ion mass information). The peptide-sequence tag program, which is part of PeptideSearch from Heidelberg, requires at least partial interpretations of the spectrum and assignment of the ions as being either *b* or *y* series. This can sometimes be difficult for PSD-MALDI-MS spectra. However, in the spectrum in **Fig. 3A**, a *y* series tag of (262.0)G(319.7) from a parent of 466.5 could be entered. It should be noted that one would normally use data from a peptide of at least eight to nine residues for database searches. A peptide that is too short will not be as useful in searches of large databases, since too many proteins will have the same (or similar) sequence. With the Heidelberg program, the search can be conducted using both ion series independently, and without constraints on potential modifications on either side of the assigned tag residue(s) (for a complete description, *see* Chapter 55). The program has detailed on-line instructions.

When a result is obtained from any of these search programs, one should attempt to assign all of the ions in the spectrum to the matched sequence. This will allow evaluation of the match and determination of whether any fragment ions are present that are not normally included in the expected ion lists generated by the search software. Regarding the peptide-mass search programs, confidence in the search results can also be gained from evaluation of the scores associated with the top-ranked matches (for those programs that have scores); e.g., if the second-ranked score is considerably less than the top-ranked score, this may be indicative of a good match. However, each program also describes how to evaluate the output. The greatest confidence (and highest score) is achieved when there are a large number of fragment ions observed that match expected ion series.

Of course, if other peptides have been observed from the same protein, even if fragment-ion spectra have not been able to be obtained from them, they can still be used to evaluate the result of the search, e.g., by determining if these other peptides could be derived from the matched protein. One should always attempt to obtain fragment-ion spectra from as many peptides in the mixture as possible to increase one's confidence in the match, and to rule out any possibility of there being more than one protein in a particular band.

4. Notes

1. Although we use a column with C8 packing, columns packed with media of differing selectivity may be more appropriate for other applications.

2. When staining the gel, **do not** use a container that has been previously used for immunoblotting protocols, e.g., for blocking with milk, and so on, since even in rinsed containers milk proteins can be adsorbed by either a blot or gel.

3. Trim the protein band carefully so that the gel piece contains only protein.

4. A control gel/membrane piece should always be included in any analysis to allow autodigestion products from the enzyme and any gel/membrane-derived ionizing species to be accounted for.

5. This step is crucial. Complete dryness must be achieved for adequate protein digestion, particularly at low pmol levels.

6. Do not add extra enzyme, since this could lead to increased autolysis and potentially spurious cleavages.

7. Add warm tap water to the sonication bath, and check the temperature prior to sonication.

8. Analysis should be performed as soon as possible, since peptides can be lost owing to adsorption to the microfuge tube, although this seems to be alleviated somewhat with the Immobilon-CD protocol possibly because of the TFA concentration.

9. Just prior to MALDI analysis, a small amount (1–5 µL) of 30% v/v MeCN/ 1% v/v HCOOH can be added to extract any peptides from the walls of the tube.

10. On lower level samples (<2.0 pmol), the majority of recovered peptides can usually (but not always) be found in the digest buffer as opposed to the acidic and organic extracts. Therefore, to increase the possibility of detecting low-level peptides in the respective extracts, these are often pooled and analyzed as one sample on the MALDI-MS. Likewise, it is advantageous to perform the MALDI-MS analysis as soon as possible to avoid again the possibility of peptides adsorbing to the walls of the tubes.

11. When excising the band, try to include only membrane that contains protein by cutting close to the edge of the band.

12. Incubation is conducted at 25°C to avoid the membrane pieces drying out owing to condensation of the buffer solution at the top of the tube. However, this is not a problem if the samples are incubated in a thermostated oven.

13. Separation of the digest buffer is not essential and is not in the original protocol *(4)*.

14. MALDI-mass spectra can be obtained from this sample directly applied to the sample well with matrix, but we have observed stronger signals, presumably owing to partial fractionation of the peptide mixture, if we first use the microcolumn to clean up the sample of salts and contaminants.

15. In addition to equilibrating the column, we suggest that a solution enzymatic digest of a known protein be used to "condition" the column prior to use. That is, load ~5 pmol of an enzymatic digest onto to the microcolumn as described in **Subheading 3.2.**, **steps 7–13**, step-elute the peptides in 30, 70, and 90% v/v MeCN/1% v/v HCOOH, and analyze by MALDI-MS. This will allow the user to become familiar with the use of the microcolumn and potentially block any nonspecific peptide binding sites in the microcolumn. We use the equivalent of a load of 5 pmol, since that is the amount we found to be retained by the C8 microcolumn *(7)*. Therefore, this method is recommended for sample loads of 5 pmol or less.

16. The volume of the wash is sample-dependent. For very dirty/salty samples, wash with an additional 20–30 μL of 1% v/v HCOOH prior to elution, or even use 5% v/v MeCN/1% v/v HCOOH.

17. The step elutions can take place at whatever MeCN concentration you desire. For this example, we use 30, 70, and 90% v/v MeCN/1% v/v HCOOH and a step volume of 3 μL.

18. In most cases, the void/wash sample will give no MALDI data owing to high-salt and contaminant concentration. If peptides are observed, this may be an indication of an overloaded microcolumn, a very old microcolumn, or peptides that do not bind to the column type chosen for the analysis. Also, if additional sensitivity is desired, the 3-μL fractions can be successfully reduced in volume with a gentle N_2 stream down to 1.0–0.5 μL.

19. Plasticizers and other contaminants can leech from the tubes over time. Be sure to run matrix only on the target well at various times to determine the mass of any contaminants. In addition, always centrifuge the matrix if there is any particulate matter (i.e., undissolved or precipitated matrix). Otherwise, the intact matrix crystals can act as crystallization seeds, causing inhomogeneous crystal formation *(24)*.

20. Even some small proteins will not ionize with 4HCCA, but will with sinapinic acid, e.g., the phosphoprotein β-casein (~24 kDa).

21. With this approach, it may be necessary to ablate several layers of the calibrant to yield signal from both calibrant and sample.

22. One can also redissolve the crystals in a small amount of additional matrix, or solvent only, if the signal has not improved.

23. The artifactual modifications characterized to date include *(1)*: cysteine-acrylamide (+71 u), oxidized acrylamide (+86 u), β-mercaptoethanol (+76 u); N-terminus acrylamide (+71 u); methionine oxidation (to sulfoxide) (+16 u).

24. It may be possible to rationalize the unassignable masses by taking into account common posttranslational modifications. However, these should always be considered tentative unless confirmatory experimental evidence is obtained.

25. Some confidence in this theory can be gained by determining whether the observed masses can be derived without assuming cleavage specificity of the enzyme (i.e., determining whether a set of contiguous residues sum to the masses observed).

26. Depending on how many masses were obtained, the masses corresponding to the matched protein can be removed and the database searched with this remaining subset of masses. This may even result in identification of the extra protein.

27. This will not occur if the database to be searched is restricted to the species of interest, but can be of assistance if the protein has not been sequenced in your species of interest. This is particularly true when working in genetically poorly characterized species.

28. This is the worst possible outcome, but one that can be interpreted (sometimes) from the difference in the scores between the first- and second-ranked candidates (i.e., if there is little difference in all of the scores, this may be a false positive).

29. Although the ionization by MALDI is said to be relatively soft, it was observed that the intact molecular ions formed undergo significant metastable fragmentation

referred to as postsource decay (PSD) *(25,26)*. This term refers to the fact that the fragmentation is thought to result from multiple early collisions between the analyte (sample) and matrix ions during plume expansion and ion acceleration (i.e., after the source), as well as from collision events in the field-free drift region of the time-of-flight analyzer. Because the metastable fragments, both neutral and charged, have the same velocity as their parent ions, they all reach the linear detector at approximately the same time. The metastable fragments are observed by decelerating ions of discrete energies as a function of their *m/z* ratios with an ion mirror, and then accelerating them back through the field-free flight-tube to a second detector. Fragment-ions have a lower kinetic energy than, but although the same velocity as their unfragmented parent ion owing to their smaller mass. These ions are resolved by lowering the potential of the ion mirror (reflector) while maintaining a constant accelerating potential. With a dual-stage reflector, this operation of decreasing the voltage (or scanning of the reflector) is performed between 7 and 14 times to generate a series of spectra that can be concatenated with appropriate software to generate a full-fragment ion spectrum. With the curved-field reflector design of Cornish and Cotter *(27)*, there is no need to step down the voltage, since all of the fragment ions are focused at once, making the process simpler and more rapid. Both types of MALDI-MS instruments have the ability to observe fragment-ion spectra from specific ions in a mixture by only allowing ions of a particular flight time into the field-free drift tube. The resolution of this gating procedure is only about ±2.5% of the parent-ion mass.

30. The predominant ion series observed by PSD-MALDI-MS, after the nomenclature of Biemann *(24)* and Roepstorff and Fohlman *(25)*, are: N-terminal-derived fragments (unimolecular cleavage with charge retained on the N-terminus): *a* and *b*; C-terminal-derived fragments (unimolecular cleavage with charge retained on the C-terminus): *y*; internal (acyl) fragments (two peptide bond cleavages) of the *a* and *b* type: referred to by the sequence of the component residues (with a –28 suffix for the *a* series type); neutral losses of ammonia (17 u, particularly strong if Arg is in the fragment of any series, and may be even more intense than the intact fragment ion) or water (18 u) from any of the previously listed ions depending on the residues contained in the sequence (particularly if Ser or Thr are in the fragment): designated as the ion series with the suffix –17 or –18; and immonium ions: designated as their single-letter abbreviation (*see* **Fig. 4** and **Table 1**). If Pro is present in the peptide, this often results in a strong internal ions series starting at the Pro and extending C-terminal from this site. For example, for a peptide of sequence ILPEFTEAR, a series of PE, PEF, PEFT, and so forth, and PE-28, PEF-28, PEFT-28, and so forth, may be observed. Other internal fragment ions are not unidirectional, but tend to cluster around basic residues present in the peptide. Whether the *a* and *b*, or *y* series ions will predominate the spectrum will depend on which terminus has the strongest basic charge, e.g., if Arg is at the C-terminus, the *y* series ions will probably predominate the spectrum. The strongest immonium ion we have observed is generated by His at 110. Even in its methylated form, as in actin *(28)*, it still yields a strong signal, in this case at 124.

31. More recently Pappin has updated the procedure, and a combination of both procedures that we have tried is presented here *(4)*.
32. Pappin *(4)* recommends using 100-µL glass-tapered vials that have been rinsed briefly in 6 *M* HCl, rinsed thoroughly with dH$_2$O, and dried at 110°C before storing in the presence of desiccant. These vials can be crimp-capped.
33. To ensure no water comes in contact with the solutions, a dry glass syringe stored with desiccant can be used. Any residual water will result in a strong reaction with the thionyl chloride, so it is recommended that all vials and measuring implements be absolutely dry.
34. Although we have generally used only 3× the volume of the original sample, Pappin *(4)* recommends using 10–15 µL of the thionyl chloride solution.

Acknowledgments

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Sample Preparation Methods for Mass Spectrometric Peptide Mapping Directly from 2-DE Gels

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1. Introduction

Polyacrylamide gel electrophoresis (PAGE) is probably the most general and widespread protein separation technique. This simple method is used in almost every molecular biology and biochemistry laboratory to monitor protein experiments, including consecutive stages of protein purification. PAGE may even be the final step of a purification protocol. Over the last decade, two-dimensional gel electrophoresis (2-D-PAGE) *(1)* has been developed, and applied to the separation of crude protein mixtures from organelles, tissues, and whole organisms *(2)*.

Methods for interfacing gel electrophoresis to "downstream" analytical techniques for protein microcharacterization and sequencing are extremely important and have been given much attention for some time. Blotting protein onto a membrane followed by amino acid analysis (Chapter 47) or by amino acid sequencing by automated Edman degradation (Chapters 48 and 49) are established methods in many laboratories. However, the sensitivity of these techniques has not been improved significantly in almost a decade. Current sensitivity limits, i.e., the required protein starting material in a gel, in state-of-the-art laboratories are 5–10 pmol for amino acid analysis and 10–20 pmol for internal Edman sequencing.

The advent of genome sequencing of model organisms reduces protein identification in those organisms to database searching with specific physicochemical protein data, such as amino acid sequence, amino acid composition, isoelectric point, molecular weight, or a combination of these features. The

ability to characterize sensitively and rapidly gel-isolated proteins would also enable the identification of individual components of purified multiprotein complexes crucial for vital biological processes, such as cell division and differentiation, signal transduction, and cell motility. However, the traditional analytical methods mentioned above either lack the sensitivity, specificity, or throughput required for analysis of many gel-isolated proteins.

Mass spectrometry (MS) has for some time been claimed to be a viable alternative to Edman degradation and amino acid analysis for low-level protein microcharacterization. However, the complexity of mass spectrometry has discouraged many protein chemists and molecular biologists from using mass spectrometry in their work. This situation is now changing owing to the commercial availability of relatively simple, but high-performance mass spectrometers equipped with matrix-assisted laser desorption/ionization (MALDI) *(3–5)* or electrospray (ES) ionization *(6,7)* sources. Improvements in MALDI and ES ion sources have resulted in dramatic improvements in sensitivity mass accuracy and mass resolution for biomolecule mass analysis *(8–13)*. It is well-established that sample preparation is a key to successful mass spectrometric analysis of biomolecules, and reproducible and robust sample preparation methods for both MALDI mass spectrometric and ES tandem mass spectrometric characterization of proteins are now available. The promise of mass spectrometry for routine protein identification and sequencing of gel-isolated proteins is finally being transformed into reality.

Over the last 3–4 yr, the main focus of research in our group has been the microcharacterization of gel-isolated proteins using analytical methods centered around mass spectrometry and bioinformatics. The techniques for sample preparation and mass spectrometric analysis of gel-isolated proteins resulting from this research are described in the following sections.

We have developed a general two-tiered strategy for mass spectrometric characterization of proteins isolated by PAGE *(14)* (**Fig. 1**). The first tier of the strategy takes advantage of the simplicity, sensitivity, and high-throughput capability of protein identification by searching protein sequence databases with a list of tryptic peptide masses derived from a protein. In-gel tryptic digestion of an excised protein band from a polyacrylamide gel results in the release and elution of peptides from the gel-matrix into the buffer solution surrounding the gel pieces. Direct analysis of an aliquot of this peptide mixture by delayed extraction MALDI time-of-flight (TOF) mass spectrometry produces a peptide map with high mass accuracy. Searching a sequence database with the list of peptide masses will retrieve the corresponding protein, if it is present in full length in the database.

If no protein is retrieved or if the data are ambiguous then the remaining peptide mixture, of which only 1–5% of supernatant is consumed by MALDI analy-

Excise protein spots, *in-gel* reduce and alkylate. (Up to 24 spots are processed in parallel)	*6 hours*

Digest *in - gel* with trypsin	*overnight*

High mass accuracy MALDI MS analysis (Analyze 0.5 μl of supernatant)	*10 min per sample*

Search database with MALDI peptide map	*10 sec per search*

IDENTIFIED? ——— YES ———→ Analyze next sample

NO

Extract gel pieces, dry down pooled extracts Perform single desalting / concentration step	*3 hours*

Nano ES tandem MS sequencing	*1 hour per sample*

Search database with peptide sequence tags	*10 sec per search*

IDENTIFIED ? ——— YES ———→ Analyze next sample

NO

Derivatize peptide mixture and re-sequence by Nano ES tandem MS. Compare two sets of tandem MS data to obtain amino acid sequences for oligonucleotide probe design and subsequent cloning .

Fig. 1. Analytical strategy for mass spectrometric characterization of gel-isolated proteins. The strategy employes high-performance MALDI peptide mass mapping for initial screening of samples. This rapid and sensitive method identifies the vast majority of known proteins. In the case of unidentified or unknown proteins, peptide sequencing by nanoelectrospray tandem mass spectrometry is pursued. Peptide sequence tags are used to screen expressed sequence tag (EST) databases. Complete peptide sequences can be generated and used in oligonucleotide design for cloning purposes.

sis, is extracted from the gel. After desalting/concentration, the peptide mixture is analyzed by nanoelectrospray tandem mass spectrometry to obtain partial peptide sequence information. A peptide sequence tag assembled from the partial sequence and the associated mass information is a specific and error-tolerant probe for database searches (Chapter 55). Two or three such sequence tags are generated from different peptides to unambiguously verify a protein identification.

If a protein turns out to be novel, then longer peptide sequences are generated by complete interpretation of nanoelectrospray tandem mass spectra

(Chapter 55) and subsequently used to design oligonucleotide probes for cloning of the protein. Generation of amino acid sequence data suitable for cloning requires somewhat higher amounts of protein than simple protein identification owing to the need to determine longer stretches of sequence and additional peptide derivatization for unambiguous sequence assignments.

In the following sections and in Chapter 55, we discuss some considerations for implementing the above strategy. Special attention is given to sample preparation methods, particularly gel staining, in-gel digestion, MALDI peptide mass mapping, and peptide sequencing by nanoelectrospray tandem mass spectrometry (Chapter 55) as used in our laboratory.

Traditionally, PAGE-isolated proteins are transferred onto a membrane by electroblotting for subsequent staining and further microcharacterization. Established methods for identification and characterization of PAGE-isolated, electroblotted proteins include antibody-labeling (Western blotting), amino acid analysis, and N-terminal Edman sequencing. Internal amino acid sequence of a protein can be obtained by *in situ* enzymatic digestion on the membrane, separation of the released peptides by RP-HPLC, followed by Edman sequencing of individual peptides *(15,16)*. More recently, mass spectrometry has been applied to the characterization of proteins blotted onto membranes. MALDI analysis of intact proteins directly from membranes is possible in some cases *(17,18)*, but has not yet been developed into a practical method. Similarly, mass spectrometry analysis of intact proteins directly from thin gel slabs *(19)* or eluted from excised gel bands into solution *(20,21)* has been demonstrated, but needs further development to be of routine use at low sample levels. Analysis of peptide mixtures released by *in situ* enzymatic digestion of blotted protein on membranes can be performed by MALDI *(22,23)*, by LC-MS *(24–26)*, and by CE-MS *(27)*. Although these methods for protein microcharacterization have been successful, they do have some limitations owing to the need for transferring a protein from gel to membrane and for performing *in situ* digestion: The transfer process is inherently inefficient and protein-dependent, and the release of peptides from a membrane is not quantitative owing to irreversible adsorption. It is therefore often necessary to pool multiple protein blots to obtain enough material for Edman sequencing or mass spectrometry. Despite recent improvements of the *in situ* digestion procedure *(28,29)*, sample losses encountered when blotting 0.1- to 5-pmol levels of gel-isolated protein often become intractable.

An alternative to blotting proteins from gels onto membranes is to use methods for handling proteins directly in the polyacrylamide gel matrix. This becomes particularly attractive for low- to subpicomole amounts of protein, because the protein is immobilized (i.e., "fixed") in the gel during most sample preparation steps, and it is therefore less vulnerable to losses and contamina-

tion. Sodium dodecyl sulfate (SDS), a detergent that is detrimental to mass spectrometry analysis, can be simply and efficiently removed from an excised protein band by repeatedly rinsing the gel plug with buffer solution.

For the purpose of internal peptide sequencing, a protein has to be proteolytically cleaved. Originally developed for Edman sequencing, the in-gel digestion procedure *(30)* has been gradually refined *(31–33)* and then adapted for MALDI and ES mass spectrometry in our laboratory *(34,35)*. The in-gel digestion procedure is applicable to subpicomole amounts of protein from single gels. Rinsing, reduction, and S-alkylation, as well as proteolytic digestion of protein are performed in the gel matrix ("in-gel") as outlined in **Subheading 3.2.** The method has also been found to be advantageous for use with standard techniques, such as HPLC/Edman degradation or LC-MS.

Coomassie brillant blue staining has been the method of choice for visualizing protein bands in gels if subsequent amino acid analysis or Edman sequencing was desired. The protocols outlined in this chapter are compatible with Coomassie-stained protein gels. As described in detail below, MALDI mass spectrometry now has the sensitivity to generate peptide maps from almost every protein band visible on a Coomassie-stained gel. One limitation of Coomassie for low-level protein visualization is its limited sensitivity, viz. more than 100 ng protein are usually needed. This corresponds to 2 pmol of a 50-kDa protein although lower protein levels can often be detected as "shadows." The more sensitive staining method employing silver has not until recently been used in protein sequencing projects because of its anticipated damaging effect on proteins. This rumor was put to rest when it was demonstrated that silver staining is a fast and nondamaging staining method for subpicomole peptide mapping and sequencing by mass spectrometry *(34)*. The silver-staining method is described in **Subheading 3.1.** Reverse staining methods, for example, employing zink/imidazole *(36)*, are also compatible with mass spectrometry and Edman sequencing.

2. Materials
2.1. Equipment

A vacuum centrifuge is needed.

2.2. Reagents

1. Fix solution: 45% MeOH, 5% acetic acid.
2. 0.02% Sodium thiosulfate solution: 20 mg of sodium thiosulfate dissolved in a total volume of 100 mL of water.
3. 0.1% $AgNO_3$ solution: 100 mg of $AgNO_3$ dissolved in a total volume of 100 mL of water.
4. 0.04% Formaldehyde/2% Na_2CO_3 solution: 40 L of formaldehyde, 2 g of Na_2CO_3 in a total volume of 100 mL with water.

5. 1% Acetic acid solution: 1 mL of glacial acetic acid added to 99 mL of water.
6. Water/acetonitrile 1:1 (v/v): 50 mL of water added to 50 mL of acetonitrile.
7. 0.1 M NH$_4$HCO$_3$ solution: 7.9 g of NH$_4$HCO$_3$ dissolved in a total volume of 100 mL of water.
8. 0.1 M NH$_4$HCO$_3$/acetonitrile (1:1) solution: 50 mL of 0.1 M NH$_4$HCO$_3$ solution added to 50 mL of acetonitrile.
9. 10 mM Dithiotreitol/0.1 M NH$_4$HCO$_3$ solution: 7.9 g of NH$_4$HCO$_3$, 1.54 g of dithiotreitol dissolved in a total volume of 100 mL of water.
10. 55 mM Iodoacetamide in 0.1 M NH$_4$HCO$_3$ solution: 1.02 g of iodoacetamide dissolved in 100 mL of 0.1 M NH$_4$HCO$_3$ solution.
11. Digestion buffer: 50 mM NH$_4$HCO$_3$, 5 mM CaCl$_2$, and 12.5 ng/µL of trypsin (e.g., Boehringer Mannheim, sequencing grade, or Promega, modified, sequencing grade).
12. 25 mM NH$_4$HCO$_3$ solution: Add 25 mL of 0.1 M solution to 75 mL of water.
13. 5% Formic acid solution: Add 5.58 mL of 88% formic acid to 94.42 mL of water.
14. Matrix solution: (saturated solution ~ 40 g/L) of α-cyano-4-hydroxycinnamic acid in acetone in a 1.5-mL microcentrifuge tube. Vortex briefly. Centrifuge to precipitate insoluble matrix material. This solution should be freshly prepared.
15. Nitrocellulose solution: Mix 50 mL of acetone with 50 mL of isopropanol. Add 1 g of nitrocellulose. This solution can be stored for several months.
16. FENC solution: Mix 4 vol of matrix solution and 1 vol of nitrocellulose solution. Addition of 1–3% (v/v) water to the FENC solution reduces the evaporation rate and thereby promotes formation of a thicker matrix surface (*see* **Note 6**).

3. Methods

   Use the purest chemicals available at all stages of sample preparation, including gel casting and staining. Avoid contamination by ionic detergents and polymers (polyethylene glycol) by thoroughly cleaning glass plates used for gel casting in methanol and water. It is advisable to let a freshly prepared gel rest overnight to reduce the amount of remaining unreacted acrylamide, which otherwise may react with thiol groups in proteins. Gloves should be worn to avoid contamination by human epidermal proteins (keratins). It is important to rinse gloves with water to remove talcum powder and traces of dust, and to inspect microcentrifuge tubes for dust particles prior to use. Contamination of gloves and tubes is often owing to electrostatic charging, which attracts dust particles.

3.1. Silver Staining of Polyacrylamide Gels for In-Gel Digestion

1. After the gel has been run, soak it for 20–30 min in fix solution.
2. Rinse the gel in water (30–60 min). Longer incubation times, e.g., 12 h, improve the contrast of the stained gel (*see* **Note 1**).
3. Sensitize the gel with 0.02% sodium thiosulfate solution for 1–2 min (**do not** use glutaraldehyde—it is a protein crosslinking agent).
4. Discard solution, and rinse the gel slab with two changes of water (1 min each).

5. Incubate the gel in chilled 0.1% $AgNO_3$ solution for 20–40 min at 4°C (in refrigerator).
6. Discard solution and rinse the gel with two changes of water (1 min each).
7. Develop gel with a solution of 0.04% formaldehyde/2% Na_2CO_3 solution on a shaking table. **Important:** Replace developing solution when it turns yellow. Do not overexpose the gel.
8. Quench development when sufficient staining is obtained (usually after 0.5–5 min) by discarding developer solution and addition of 1% acetic acid.
9. Store the silver-stained gel in 1% acetic acid or in water at 4°C.

3.2. In-Gel Protein Digestion

This protocol *(34)* is applicable without any modification to one-dimensional as well as two-dimensional polyacrylamide gels of varying thickness (routinely 0.5–1.5 mm), acrylamide concentration (7–18% of acrylamide), and protein band (spot) size. Gels are prepared by standard techniques using 0.1% SDS. Proteins are visualized by staining with Coomassie brilliant blue R-250 or G-250, or alternatively by silver staining (**Subheading 3.1.**) or reverse staining (zinc-imidazole staining) (Chapters 33 and 34 of this volume) *(36)*. The extensive washing steps preceding reduction and S-alkylation can be omitted if the silver-staining technique is used.

3.2.1. Excision of Protein Bands from Polyacrylamide Gels

1. Wash the stained gel slab with water (2 changes, 10 min each).
2. Use a clean scalpel to excise the spot of interest from the gel. Cut as close to the protein band as possible to reduce the amount of "background" gel. Excise a gel piece of roughly the same size from a nonprotein-containing region of the gel for use as a control (*see* **Note 2**).
3. Cut the excised piece into roughly 1-mm³ cubes, and transfer them to a clean 1.5- or 0.5-mL microfuge tube.

3.2.2. Washing of Gel Pieces

1. Wash the gel particles with water and water/acetonitrile 1:1 (v/v) (one or two changes each, 15 min/change). Solvent volumes used in the washing steps should roughly equal two times the gel volume.
2. Remove all liquid, and add enough acetonitrile to cover the gel particles.
3. After the gel pieces have shrunk (they become white and stick together) remove the acetonitrile, and rehydrate the gel pieces in 0.1 *M* NH_4HCO_3 for 5 min.
4. Add an equal volume of acetonitrile (to get 0.1 *M* NH_4HCO_3/acetonitrile, 1:1), and incubate for 15 min.
5. Remove all liquid, and dry down gel particles in a vacuum centrifuge.

3.2.3. Reduction and Alkylation

1. Swell the gel particles in 10 m*M* dithiotreitol/0.1 *M* NH_4HCO_3, and incubate for 45 min at 56°C to reduce the protein. We recommend in-gel reduction even if the proteins were reduced prior to an electrophoresis run.

2. Chill tubes to room temperature. Remove excess liquid, and replace it quickly with roughly the same volume of freshly prepared 55 mM iodoacetamide, 0.1 M NH$_4$HCO$_3$ solution.

3. Incubate for 30 min at room temperature in the dark.

4. Remove iodoacetamide solution, and wash the gel particles with 0.1 M NH$_4$HCO$_3$ and acetonitrile as described in **Subheading 3.2.2.** All the Coomassie stain should be removed at this time. The gel particles should appear completely transparent. If a large amount of protein is analyzed (more than 10 pmol), residual Coomassie staining may still be observed. In this case, an additional 0.1 M NH$_4$HCO$_3$-aceto-nitrile washing cycle should be performed. When the silver-staining method is used, a single rinsing cycle suffices.

3.2.4. In-Gel Digestion (*see* **Note 3**)

1. Gel particles are completely dried down in a Speed Vac. (If a large gel volume is analyzed, shrink it by addition of acetonitrile before drying.)

2. Rehydrate gel particles by a digestion buffer at 4°C (ice bucket) (*see* **Note 3**). Add enough digestion buffer to cover the gel pieces. Add more buffer if all the initially added volume is absorbed by the gel pieces. Incubate for 45 min on ice.

3. Remove remaining enzyme supernatant, and replace it with 5–20 µL of the same buffer—but without enzyme—to keep the gel pieces wet during enzymatic cleavage (37°C, overnight).

3.2.5. Direct MALDI Mass Analysis of Released Peptides

1. The first peptide analysis can be performed already after 3–4 h of digestion. If some liquid has evaporated and condensed on the side or on the lid of the microcentrifuge tube, centrifuge briefly to gather the liquid at the bottom of the tube.

2. Wait for 15 min, vortex briefly, and then remove 0.5 µL of the supernatant and mix it into a 0.5-µL droplet of 10% formic acid previously deposited onto a fast evaporation matrix surface with nitrocellulose (*see* **Subheading 3.3.**). Acquire a MALDI peptide mass map, and then search a protein sequence database with the list of peptide masses as described below.

3.2.6. Extraction of Peptides from Gel

1. After overnight digestion, add a sufficient volume of 25 mM NH$_4$HCO$_3$ to cover the gel pieces and incubate for 15 min.

2. Add the same volume of acetonitrile. Incubate for 15 min, and recover the supernatant.

3. Repeat the extraction two times with 5% formic acid and acetonitrile (1:1, v/v).

4. Pool all the extracts.

5. (Optional) add 10 mM dithiotreitol solution to a final concentration of 1 mM dithiotreitol in the total sample volume. This reduces oxidation during storage.

6. Dry the sample in a vacuum centrifuge.

3.2.7. Mass Spectrometric Peptide Mapping and/or Sequencing

1. Redissolve the peptides in 10–30 µL of 5% formic acid, sonicate briefly, and analyze an aliquot by MALDI mass spectrometry.

2. The recovered peptide mixture can be directly injected and analyzed by HPLC or by HPLC-ES MS if the sample amount suffices. Use a desalting/concentration microcolumn prior to low-level peptide sequencing by nanoelectrospray tandem mass spectrometry (*see* Chapter 55).

3.3. Sample Preparation for MALDI Peptide Mass Mapping

MALDI mass spectrometry has several features that makes it an excellent method for initial screening of peptide mixtures generated by in-gel digestion of a protein band (*see* **Note 4**). We routinely use the fast evaporation method *(37,38)* for preparing matrix surfaces of α-cyano-4-hydroxycinnamic acid *(5)* for MALDI peptide mass mapping. This procedure allows the matrix and the sample to be applied onto the MS probe in independent steps, i.e., the sample solution can be optimized independent of the matrix solution. It simplifies sample preparation, and also improves sensitivity, resolution, and mass accuracy attainable in MALDI peptide analysis. An improved rugged matrix surface is prepared by mixing nitrocellulose into the matrix solution *(34,39)*. This method is tolerant to salts, reagents, and buffers present in peptide mixtures generated by in-gel digestion.

1. Prepare a saturated matrix solution (*see* **Subheading 2.2.** and **Note 5**).
2. Cut out a piece of nitrocellulose membrane (Bio-Rad Transblot medium), and make a nitrocellulose solution.
3. Prepare a fast evaporation/nitrocellulose (FENC) solution.
4. Deposit 0.2–0.3 μL FENC solution by rapid transfer from the microcentrifuge tube to the MALDI MS probe (use a 2-μL adjustable pipet) (*see* **Note 6**).

3.3.1. Direct Analysis
of Peptide Containing Supernatant After In-Gel Digestion

1. Deposit 0.5 μL of 10% formic acid on an FENC matrix surface.
2. Remove 0.5-μL supernatant from the in-gel digested protein sample, and inject it into the droplet. This approach ensures that the final peptide solution remains acidic (pH < 4.0), so that the matrix film does not completely redissolve owing to the presence of 50 m*M* ammonium bicarbonate in the supernatant.

3.3.2. Analysis of Extracted Peptides After In-Gel Digestion

1. Extract the peptides after in-gel digestion as outlined in **Subheading 3.2.6.**
2. Redissolve the dried peptide sample in 10–30 μL of 5% formic acid. Sonicate and vortex briefly. Deposit 0.5–1 μL of this peptide solution onto an FENC surface.
3. Rinse the FENC surface by depositing 10–20 μL of 5% formic acid followed by immediate wiping or shaking of the probe to remove the liquid. Repeat the rinsing step with 10–20 μL of pure water. The rinsing procedure can be repeated several times provided that the matrix film is not damaged.
4. Insert the probe into the MALDI mass spectrometer, and acquire a spectrum (*see* **Note 7**).

5. Perform a database search using acquired spectrum (*see* **Note 8**).
6. Confirm database hit (*see* **Note 9**).
7. For identifying large numbers of 2-D PAGE spots, an automated strategy is implemented (*see* **Note 10**).
8. Evaluate data to determine if nanospray ESI analysis (Chapter 55) should be used for peptide sequencing (*see* **Note 11**).

4. Notes

1. For highest sensitivity, rinse the gel slab in water for at least 60 min after the gel has been run and fixed. This helps to keep the background transparent during development of the silver stain. Prepare fresh solutions of reagents. For more details, refer to **ref.** *34*. Note that prestained mol-wt-markers are not suitable for silver staining, because they become overstained.
2. If the protein of interest appears to be contaminated with other proteins migrating nearby, then excise the control blank below the band of interest to obtain a representative pattern of contaminations.
3. Trypsin is the enzyme of choice, because it generates peptides in the mass range of 800–2500 Dalton, which is optimal for MALDI peptide mass mapping and electrospray tandem mass spectrometric sequencing. The in-gel digestion procedure works equally well with other enzymes.
4. Sample preparation for MALDI is exceedingly simple, particularly when using the fast evaporation matrix deposition method *(37,38)*. Addition of nitrocellulose to the matrix solution gives even better performance and tolerance to contaminants *(34,39)* (**Subheading 3.2.**). MALDI is often combined with a TOF mass spectrometer that provides very high sensitivity because all molecular ions are mass-analyzed and detected in one experiment—no scanning mass analyzer is involved. In our laboratory, the sensitivity of MALDI reflector TOF mass spectrometry for peptide mass mapping applications is routinely in the low-femtomole range. This is sufficient for analysis of aliquots of peptide mixtures generated by in-gel digestion of protein bands from Coomassie and silver-stained gels. We have found that direct MALDI analysis of peptide supernatant from an in-gel digestion solution provides better signal-to-noise ratio compared to analysis of the extracted, vacuum-dried, and redissolved peptide mixtures (*see* **Subheading 3.3.**). Measurement of peptide aliquots after 3–4 h of in-gel digestion already generates high-quality peptide mass maps. Thus, the identification of a set of proteins can be achieved in a few hours. Although the excellent sensitivity of MALDI is well-established, the technique has been of limited use for peptide mass mapping for protein identification mainly because of poor mass accuracy and low mass resolution achievable by most commercially available TOF instruments. The general problem of low mass accuracy in TOF mass spectrometry has now been remedied by the introduction of time-lag-focusing, a.k.a., delayed extraction ion sources for MALDI *(40–43)*. Delayed extraction allows the MALDI-generated desorption plume to dissipate before molecular ions are extracted into the TOF mass analyzer by a pulsed electric field. The reduced

number of gas-phase ion collisions generates only a minimal ion kinetic energy spread, which in turn provides very high peak resolution and improved signal-to-background ratio. A simple linear TOF analyzer can resolve the isotopes of small peptide ions generated by delayed-extraction MALDI. Peak resolution (full-width half-maximum) in the range of 8000–15,000 is achievable when employing a reflector TOF analyzer. The improved mass resolution makes peptide mass assignments easier, because the monoisotopic mass can be determined even for low-intensity ion signals. Mass calibration employing known "background ion" signals improves peptide mass accuracy to 30 ppm or better over the *m/z* range of 800–3000 *(39,44)*. As discussed below, this is important for unambiguous protein identification by MALDI peptide mass maps.

5. α-Cyano-4-hydroxycinnamic acid and sinapinic acid are routinely used to prepare fast evaporation surfaces, but ferulic acid and 2,4,6-trihydroxyacetophenone can also be employed.

6. It is important that the droplet can spread out (no confining edges on the probe) and dry in a matter of 1–2 s. The resulting thin matrix film should appear homogenous and yellowish. No crystals should be visible to the naked eye. Depending on the particular MALDI MS probe design, it may be necessary to optimize conditions by varying the concentration of matrix, nitrocellulose, or water in the acetone solution or the volume of liquid applied onto the MS probe.

7. Internal mass calibration of MALDI TOF spectra is desirable in order to obtain the highest possible peptide mass accuracy. We routinely use trypsin autodigestion peptide signals and matrix ion signals inherent to every MALDI peptide mass map generated by the procedures outlined in **Subheadings 3.2.** and **3.3. (39,44)**. Trypsin autolytic peptides are produced in the required amount owing to the relatively high amount of enzyme used for in-gel digestion (**Subheading 3.2.**). MALDI analysis of a trypsin autodigest solution reveals the autolytic peptides for a given trypsin batch. Unmodified (nonalkylated) trypsin has the advantage that all autolytic peptide ions can be assigned to the trypsinogen amino acid sequences available in databases. Matrix ion signals are recognized by analysis of a "blank" sample applied to the matrix surface. In peptide mass maps, matrix ion signals in the *m/z* range of 800–1100 are recognized by their fractional mass values that differ from peptide fractional mass values. An example of a MALDI peptide mass map calibrated with this method is shown in **Fig. 2**. The measured peptide masses map to the cognate protein sequence (S-adenosylmethionine synthetase 2; SwissProt P19358) to within 30 ppm. In spectra where no matrix or autolytic peptides are apparent, either external calibration or internal calibration by addition of peptide standards has to be employed.

8. Searching a protein sequence database by peptide mass maps is, in principle, a simple method for protein identification *(45–49)*: A set of measured tryptic peptide masses is searched against all sets of predicted tryptic peptide masses for protein sequences in a database. In theory, no other information, such as species, protein molecules weight, and protein p*I*, is needed to identify a protein. In practice, however, the mass mapping approach was hampered by the low mass accu-
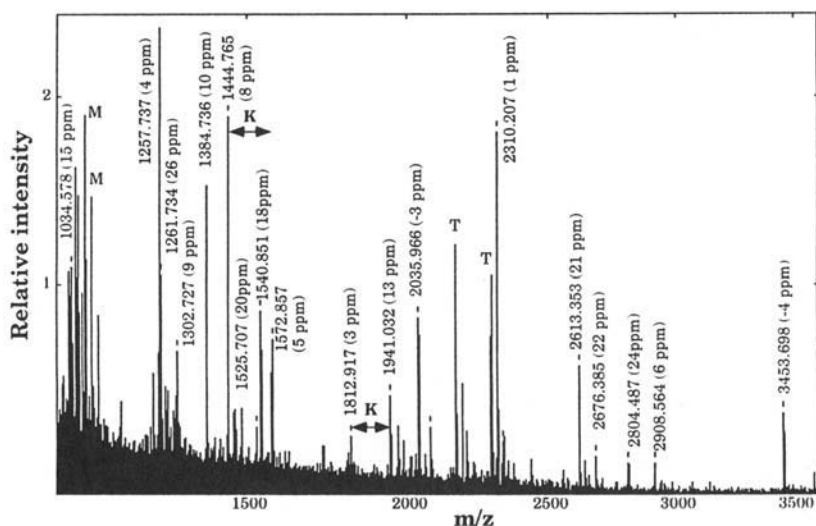
Fig. 2. Delayed extraction MALDI mass map obtained after in-gel digestion of a silver-stained protein spot from a 2-D gel. An aliquot consisting of 0.3 µL supernatant from in-gel digestion of the protein was deposited on a fast evaporation film of matrix and nitrocellulose. The peptide mixture was analyzed by automated MALDI *(53)* and the list of peptides was automatically searched in a protein database. Yeast S-adenosylmethionine synthetase 2 (SwissProt P19358) was unambiguously identified. The 18 matching peptides and their mass deviation from the calculated masses are labeled in the spectrum. Two terminal peptide sequence tags *(52)* owing to adjacent tryptic cleavage sites are labeled with arrows. Matrix-related ion signals (M) and trypsin autodigestion peptides (T) are indicated. Based on the intensity of staining and the ion intensity in the mass spectrum, the protein amount in the gel was estimated to be 1–2 pmol.

racy, typically 1–2 Dalton, which was until recently attainable by most commercial MALDI instruments. The accuracy of peptide mass determination has a dramatic effect on the search specificity when querying a comprehensive protein sequence database *(44,50)*. This is particularly so when <10 peptides are detected or when protein mixtures are encountered. Searching with a mass tolerance of ±1 Dalton then becomes impractical because it leads to too many random protein matches. Our strategy has therefore been to improve mass accuracy as much as possible to increase search specificity. Protein origin, molecular weight, or p*I* are not used as search parameters in the primary database query, because they may serve to exclude correct protein identification in the cases of protein contaminations or protein posttranslational modification. For example, human proteins (keratins) are often detected in yeast protein preparations, and protein processing can generate a small polypeptide from a large precursor protein *(14,51)*. In our experience, it is necessary to measure peptide masses with a mass accuracy of 30–100 ppm for unambiguous database searching applications *(14,39,44,51)*. The

introduction of delayed extraction for MALDI has made peptide mass determination within 50 ppm routinely achievable. This high mass accuracy increases the database search specificity of peptide mass maps, and it is now feasible to identify a protein with certainty even when only four to six peptides are detected *(39)*. This becomes increasingly important for identification of subpicomole levels of gel-isolated protein by MALDI MS, because few peptides are detected. Furthermore, the high peptide mass accuracy enables protein mixtures in a single gel band to be resolved by iterative database searches *(44)*. Such comigrating proteins are often present in one-dimensional gel electrophoresis of complex protein mixtures. Several database search programs are available as stand-alone applications or as Internet services. A list of the most popular programs is available via our homepage at http://www.mann.embl-heidelberg.de/. In our laboratory, the *PeptideSearch* program *(46,50)* is used to query a nonredundant protein database that currently contains more than 260,000 entries.

9. A number of criteria are used to evaluate protein identifications made by MALDI peptide mass maps and database searches. If the majority of measured tryptic peptide masses map to a protein sequence to within 50 ppm, then the protein identification is unambiguous. Since a comprehensive protein sequence database is searched, the species of origin, the M_r, and the pI determined by the 2-D gel separation of a protein serve to confirm independently an assignment after the database search has been performed. Additionally, a protein identification is evaluated by the sequence coverage of the detected peptides, i.e., the number of amino acids covered by the measured tryptic peptides relative to the total number of amino acids in the retrieved protein. In most cases, we require the sequence coverage to be better than 15% to call an unambiguous identification. However, for proteins larger than 100 kDa, the sequence coverage can be below 10% and still allow 15–20 tryptic peptides to match the sequence within 50-ppm accuracy and, therefore, uniquely identify the protein. For proteins below 20 kDa, as few as three to four tryptic peptides may be detected, which nevertheless amount to 30–50% sequence coverage and therefore suffice to identify the protein. The presence of a methionine-containing peptide may be confirmed by the appearance of a satellite peak 16 Dalton higher in mass caused by an oxidized methionine residue (methionine sulfoxide). Samples that have been stored may exclusively contain oxidized methionine. Peptide mass maps should always be inspected for the presence of cysteine-containing peptides which are S-alkylated with acrylamide owing to the presence of unreacted reagent in gels. This is often the case when separating proteins on freshly prepared gels. The N-terminal domain of a protein is usually accessible to the protease, so the N-terminal tryptic peptide may be displayed in peptide mass maps. If it is not detected at the mass calculated from the amino acid sequence, then try to remove the initiating methionine residue or allow for *N*-acetylation or other common posttranslational modifications of the N-terminus. Inspection of a sequence database entry for the retrieved protein often reveals if it is known to be posttranslationally modified and helps in the assignment of additional peptide ion signals. We have noted that tryptic cleavage

sites may occur in tandem in proteins, viz. as Lys-Lys, Arg-Lys, Lys-Arg, or Arg-Arg sequence patterns *(52)*. Tryptic cleavage at these sites usually generates two peptides differing in only a Lys or Arg residue. Both of these peptides are often detected: Inspection of more than 50 MALDI peptide mass maps of yeast and vaccinia virus proteins revealed that these patterns occur in about half of the peptide mass maps and that they can be easily detected by searching a mass spectrum for peptide ion signals separated by 128.1 Dalton (Lys) or 156.1 Dalton (Arg). The presence of "terminal peptide sequence tags" in a spectrum provides additional evidence for a correct identification if the corresponding peptide with a tandem cleavage site is also found in the retrieved protein sequence. Searching a database with a terminal sequence tag and the complete peptide mass map reduces the number of randomly matching proteins significantly. Generation of multiple N-terminal sequence tags using one cycle of Edman chemistry on the complete peptide mixture is also possible *(52)*.

10. To realize the potential of MALDI peptide mass mapping for high-throughput protein identification, we have developed an automated system that allows unattended MALDI data acquisition, mass calibration, peak annotation, and protein database searching *(53)*. The crucial parameter in MALDI data acquisition, attenuation of the UV laser fluence to optimize spectrum quality, is performed by a fuzzy logic feedback control system, which monitors the peptide ion intensity and mass resolution in real time. These input parameters are evaluated by a set of fuzzy rules and fuzzy membership functions, which immediately determine the required adjustment of laser attenuation and the movement of the probe relative to the laser beam. Algorithms for mass calibration, peak annotation, and database searching with a list of peptide masses have also been implemented. The MALDI peptide mass map shown in **Fig. 2** was automatically acquired and processed. The automated MALDI system in the current configuration can process 10–12 protein samples/h. Combined use of a robotic sample preparation workstation for in-gel digestion *(54,55)* and automated MALDI for protein identification is a promising method for high-throughput protein identification *(53)*.

11. Although MALDI peptide mass mapping and database searching is an attractive and efficient method for protein identification, it does have some limitations. Detection of at least four to five peptides from a protein is required to identify the latter with certainty. This is not always possible, e.g., in the cases of some small basic proteins or some hydrophobic proteins. Low amounts of protein in a gel band, i.e., <1 pmol, reduces the number of extracted and detectable peptides after in-gel digestion. Most importantly, the method relies on the near-complete protein sequence being present in a sequence database. With the increasing amount of DNA sequence data accumulated by concerted genome sequencing efforts, this will not be a major restriction. We are currently able to identify a majority of proteins from common model organisms (human, mouse, rat, fruit fly) and more than 90% of yeast proteins by MALDI peptide mass mapping and database searching. In the case of novel proteins, the data quality obtained by MALDI peptide mass mapping in our laboratory is sufficient to determine that a protein is

positively unknown and thereby guide the researchers in their decision to perform *de novo* peptide sequencing. Reflector TOF mass spectrometry can be used for peptide sequencing by analysis of metastable decay products. Peptide sequencing by postsource decay (PSD) analysis *(56)* by MALDI reflector TOF is described in detail in Chapter 51. Unfortunately, we have found MALDI mass spectrometry in its current state to be of limited use for low-level peptide sequencing by PSD analysis because it is time consuming, the peptide fragment ions are of low intensity, and PSD spectra are difficult to interpret. This is also the case for "ladder sequencing" of peptide mixtures by carboxy- or aminopeptidases or by chemical methods. Instead, we have focused on methods for peptide sequencing by nanoelectrospray tandem mass spectrometry on a triple-quadropole instrument. These methods are outlined in Chapter 55.

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53

Protein Identification and Analysis Tools in the ExPASy Server

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1. Introduction

Protein identification and analysis software performs a central role in the investigation of proteins from two-dimensional (2-D) gels. For protein identification, the user matches certain empirically gained information against a protein database to define a protein as already known or as novel. For protein analysis, information in protein databases can be used to predict certain properties about a protein, which can be useful for its empirical investigation. The two processes are thus complementary. Although there are numerable programs available for the above-mentioned applications, we have developed a set of original tools with a few main goals in mind. Specifically, these are:

1. To utilize the extensive annotation available in the SWISS-PROT database *(1)* wherever possible.
2. To have tools specifically, but not exclusively, applicable to proteins prepared by 2-D gel electrophoresis.
3. To have all tools available on the World Wide Web and freely available to the scientific community.

This chapter gives details about protein identification and analysis software, which is available through the ExPASy World Wide Web server *(2)*. These include Compute pI/Mw, a tool for predicting protein isoelectric point (p*I*) and molecular weight (Mw) in a 2-D gel; PeptideMass, a tool for theoretically cleaving proteins and calculating the masses of their peptides and any known cellular or artifactual posttranslational modifications; TagIdent, a tool that lists

proteins within a user-specified p*I* and mol-wt region, and allows proteins to be identified through the use of short "sequence tags" of up to six amino acids in length; AACompIdent, a program that identifies proteins by virtue of their amino acid (AA) compositions, sequence tags, p*I* and molecular weight; AACompSim, a program that matches the theoretical AA composition of proteins against the SWISS-PROT database to find similar proteins; and MultiIdent, a combination of other tools mentioned above that accepts multiple data types to achieve identification, including protein p*I*, molecular weight, species of interest, AA composition, sequence tag, and peptide masses.

The tools described here are continually under development and thus may change with time. We document new features of tools in the "What's new on ExPASy" World Wide Web page at URL address: http://www.expasy.hcuge.ch/www/history.html. We welcome feedback and suggestions from users of the tools. Check the "Geneva Proteome Project Team" World Wide Web page at http://www.expasy.hcuge.ch/www/people.html to see the people working on ExPASy, and send any comments to us by e-mail.

2. Materials

The tools described here are accessible through the ExPASy World Wide Web server *(2)*. To access this server, you require a computer connected to the internet, such an Apple Macintosh, a Windows-based PC, or a Unix X-Windows system, and a World Wide Web browser, such as Netscape™. If you are a student, staff member, or faculty member of an educational institution, you can download and use a copy of Netscape™ Navigator free of charge. Look at this URL address for further details: http://home.netscape.com/comprod/mirror/index.html.

Since many of our tools run in batch mode and send results by electronic mail, you will also need an e-mail address. Your institute's computer systems manager can arrange for one of these to be issued. Finally, to connect to the Tools menu of the ExPASy server, go to the URL address of: http://www.expasy.hcuge.ch/www/tools.html where you should see the menu as in **Fig. 1**.

3. The Tools
3.1. Introductory Comments

The tools below all work directly and exclusively with the SWISS-PROT annotated protein database, but are currently being modified also to work with the TrEMBL supplement to SWISS-PROT *(1)*. Unless otherwise stated, the tools use SWISS-PROT annotations to process polypeptides to their mature forms before using them for calculations or protein-identification procedures. Thus, protein signal sequences and propeptides are removed where found, and precursor molecules processed into their resulting chains. However, it should be noted that there are many hypothetical proteins in the database resulting
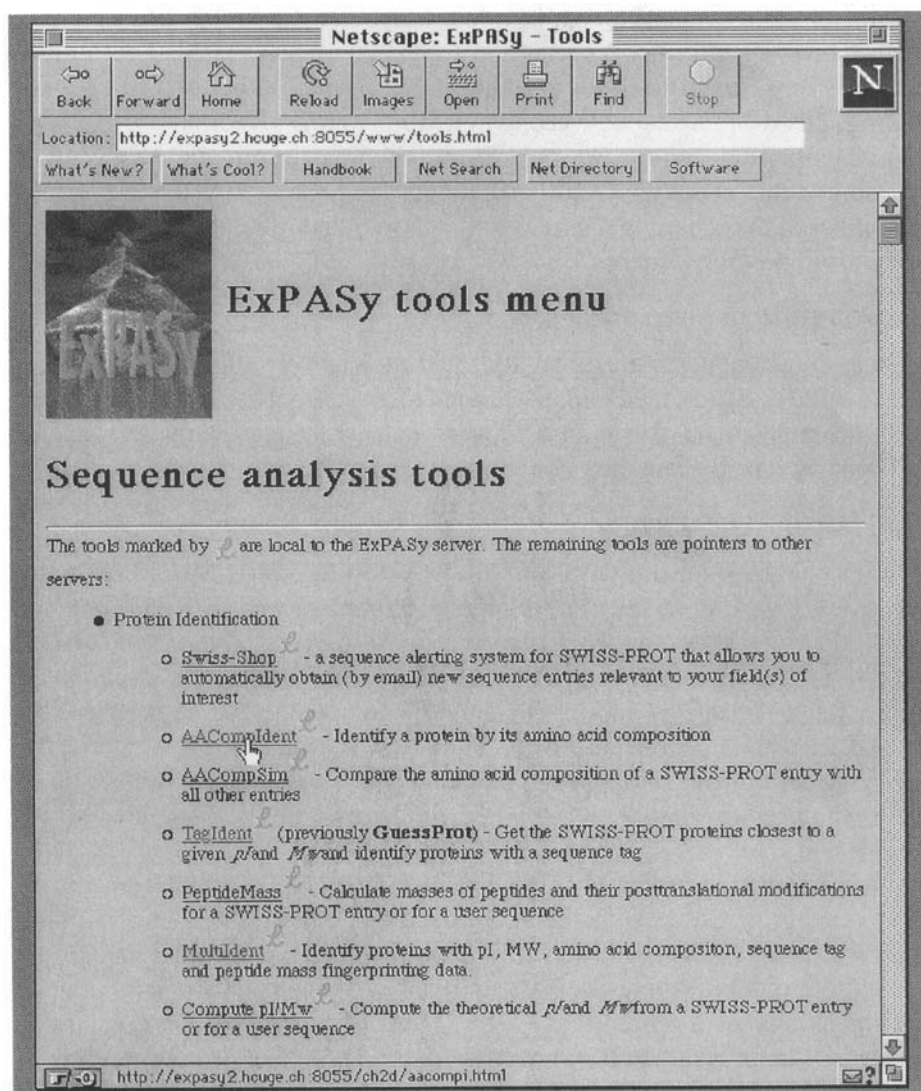
Fig. 1. The ExPASy Tools page, as accessed with the Netscape™ World Wide Web navigator. All underlined text represents hypertext links, which when selected with a computer mouse take the user to the corresponding page for the chosen tool.

from genome projects, which may have little or no annotation. It should also be noted that unless stated otherwise, the tools below do not account for known posttranslational modifications (e.g., glycosylation, phosphorylation, acetylation) in calculation of protein p*I* and molecular weight.

When entering sequence data into text boxes for the tools, note that any spaces, newline (return) characters, and numbers will be ignored. This allows sequences in other formats, for example, GCG format, to be used directly in the programs without first converting to text-only (ASCII) format.

The numbering used by the tools for amino acids in protein sequences is according to that in SWISS-PROT. If proteins are processed to mature forms, the number of the N-terminal amino acid will remain the same as it was in the unprocessed protein sequence.

3.2. Compute pI/Mw Tool

This tool calculates the estimated p*I* and molecular weight of a specified SWISS-PROT/TrEMBL entry or a user-entered AA sequence (*see* **Notes 1** and **2**). These parameters are useful if you want to know the approximate region of a 2-D gel where a protein may be found.

3.2.1. Using Compute pI/Mw

To use the program, enter one or more SWISS-PROT/TrEMBL identification names (e.g., LACB_BOVIN) or accession numbers (e.g., P02754) into the top text field, and select the "click here to compute pI/Mw" button (*see* **Note 3**).

If one entry is specified, you will be asked to specify the protein's domain of interest for which the p*I* and mass should be computed. The domain can be selected from the hypertext list of features shown, if any, or by numerically specifying the domain start and end points.

If more than one SWISS-PROT/TrEMBL identification name is entered, all proteins will automatically be processed to their mature forms, and p*I* and mol-wt values calculated for the resulting chains or peptides. If only fragments of the protein of interest are available in the database, no result will be given, and an error message will be shown to highlight that the p*I* and mass cannot be returned accurately. Where database entries have signal sequences or transit peptides of unknown length (e.g., ATPI_ODOSI), an average length signal sequence or transit peptide is removed before the p*I* and mass computation is done. In SWISS-PROT release 35, the average signal sequence length is 22 amino acids for eukaryotes and viruses, 26 amino acids for prokaryotes and bacteriophages, and 30 for archaebacteria. Transit peptides have an average length of 55 amino acids in chloroplasts, 34 for mitochondria, 29 for micro-bodies, and 51 for cyanelles.

If your protein of interest is not in the SWISS-PROT database, you can enter an AA sequence in standard single-letter AA code into the second (lower) text field, and select the "click here to compute pI/Mw" button. The predicted p*I* and molecular weight of your sequence will then be displayed. A typical output from the program is shown in **Fig. 2**.

```
LACB_BOVIN (P02754)

DE    BETA-LACTOGLOBULIN PRECURSOR (BETA-LG).
OS    BOS TAURUS (BOVINE).

The parameters have been computed for the following feature:
FT    CHAIN        .17    178        BETA-LACTOGLOBULIN.

Considered sequence fragment:
LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP EGDLEILLQK
WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK KYLLFCMENS AEPEQSLACQ
CLVRTPEVDD EALEKFDKAL KALPMHIRLS FNPTQLEEQC HI

Molecular weight: 18281.00

Theoretical pI: 4.83
```

Fig. 2. Sample output from the Compute pI/Mw tool, where the program was requested to calculate the theoretical p*I* and molecular weight for the SWISS-PROT entry LACB_BOVIN (P02754). Note that the Compute p*I*/Mw tool shows the sequence of the region of the protein that is under consideration. In this case, the sequence of the mature β-lactoglobulin is shown, which results when the secretion signal sequence is removed from the precursor polypeptide.

3.3. PeptideMass Tool

The PeptideMass tool is designed to assist in peptide mapping experiments, and in the interpretation of peptide mass fingerprinting results and other mass spectrometry data *(3)* (*see* **Note 4**). It theoretically cleaves user-specified protein sequences or mature proteins in the SWISS-PROT/TrEMBL databases with an enzyme or reagent of choice to generate peptides. Masses of the peptides are then calculated and displayed (*see* **Note 5**). If a protein from SWISS-PROT has annotations that describe discrete posttranslational modifications (specifically acetylation, amidation, biotinylation, C-mannosylation, formylation, farnesylation, γ-carboxy glutamic acid, geranyl-geranylation, lipoyl groups, *N*-acyl glycerides, methylation, myristoylation, NAD, *O*-GlcNAc, palmitoylation, phosphorylation, pyridoxyl phosphate, pyrrolidone carboxylic acid, or sulfatation), the masses of these modifications will be considered in peptide mass calculations (*see* **Note 6**). The mass effects of artifactual protein modifications, such as the oxidation of methionine or acrylamide adducts on cysteine residues, can also be considered. The program can supply warnings where peptide masses may be subject to change from protein isoforms, database conflicts, or mRNA splicing variation (*see* **Note 7**).

3.3.1. Using PeptideMass

To use the program, enter one or more SWISS-PROT identification names (e.g., TKNB_HUMAN) or any SWISS-PROT/TrEMBL accession numbers (e.g., P20366) into the text field, or enter a protein sequence of interest using

the standard one-letter AA code. User-specified sequences should not contain the character "X," but can contain the character "J" to represent either Ile or Leu, which are of the same mass. The enzyme or reagent to use to cleave the protein sequence theoretically should then be specified, and if any missed cleavages should be allowed. Special treatment (if any) of cysteine residues or oxidation of methionine should be selected, and if results are desired, as monoisotopic or average masses. Finally, click on the "Perform" button to send data to the program. The results should arrive immediately, but note that if many options have been specified, it may be necessary to scroll to the right to see the entire program output. **Figure 3** shows a typical output of the program PeptideMass, illustrating some of its features.

3.4. TagIdent Tool

The TagIdent tool *(4)* serves two main purposes. First, it can create lists of proteins from one or more organisms that are within a user-specified p*I* or mol-wt range (*see* **Note 8**). This is useful to find proteins from the database that may be in a region of interest on a 2-D gel. Second, the program can identify proteins from 2-D gels by virtue of their estimated p*I* and mol wt, and a short protein "sequence tag" of up to six amino acids. The sequence tag can be derived from protein N-termini, C-termini, or internally, and generated by chemical or mass spectrometric sequencing techniques. Since sequence tags are highly specific (e.g., there are 160,000 different combinations of four amino acid sequence tags), they represent a form of protein identification that is useful for organisms that are molecularly well defined and have a relatively small number of proteins (e.g., *Escherichia coli* or *Saccharomyces cerevisiae*). Interestingly, we have shown elsewhere that C-terminal sequence tags are more specific than N-terminal tags *(4)*. However, it remains technically more difficult to generate high-quality C-terminal protein sequence data.

3.4.1. Use of TagIdent to List Proteins in a Defined p*I* and/or Mol-Wt Region

To use TagIdent to generate a list of proteins in a p*I* and mol-wt range of interest, you must first specify a full e-mail address to where the results can be sent. If desired, a name can be given to the query, which will appear as the subject of the e-mail message. This is useful for archiving purposes or if many different queries are to be submitted to the program at the same time. You should then specify the p*I* and mol-wt regions within which you would like to search (e.g., p*I* of 5.5 ± 0.5 U and mol wt 20000 ± 10%). If you would like to search using only one of the p*I* or mol-wt parameters, you can specify an unrestricted window to cover all possibilities for the other parameter (*see* **Note 9**). For example, a search where p*I* is set to 7.0 ± 10 U, but where a mol-wt win-

```
You have selected TKNB_HUMAN from SWISS-PROT:
The selected enzyme is: Trypsin
All cysteines in reduced form.
Methionines have not been oxidized.
Using average masses of the occurring amino acid residues and giving
peptide masses as [M+H]+.

PROTACHYKININ BETA PRECURSOR (CONTAINS: SUBSTANCE P, NEUROKININ A
(SUBSTANCE K) (NEUROMEDIN L), AND NEUROPEPTIDE K) (BETA-PPT).


Peptide SUBSTANCE P at positions 58 - 68
[Theoretical pI: 11.00 / Mw: 1348.63]

  mass     position   modified   modifications peptide sequence
                        mass
 1349.64   58-  68    1348.66      AMID: 68      RPKPQQFFGLM


Peptide NEUROPEPTIDE K at positions 72 - 107
[Theoretical pI: 8.40 / Mw: 3981.51]

  mass     position   modified   modifications peptide sequence
                        mass
 1211.37   86-  96                              ALYGHGQISHK
 870.01   100- 107    869.03       AMID: 107    TDSFVGLM
 864.88    72-  79                              DADSSIEK
 671.86    80-  85                              QVALLK
 284.34    98-  99                              HK
 175.21    97-  97                              R


Peptide NEUROKININ A (SUBSTANCE K) at positions 98 - 107
[Theoretical pI: 6.74 / Mw: 1134.32]

  mass     position   modified    modifications  peptide sequence
                        mass
 870.01   100- 107    869.03        AMID: 107     TDSFVGLM
 284.34    98-  99                                HK
```

Fig. 3. Sample output from the PeptideMass tool. The protein selected was TKNB_HUMAN, and the program was requested to cleave with trypsin, show all peptides, sort peptides by mass, show all known modifications, use average masses, and display masses as $(M + H)^+$. The figures in the "modified mass" column in this case show the predicted masses of peptides known to be amidated. Note that there are three lists of peptides that correspond to the cleavage of different products known to be created from the same initial polypeptide. Underlined text and numbers represent hypertext links in the output, which, if selected, show either the SWISS-PROT entry for the protein (e.g. TKNB_HUMAN) or the sequence of any portion of a protein specified with numbers (e.g., 58–68).

dow of 20,000 ± 10% is used will return all proteins of sizes 18,000–22,000 mol wt, regardless of their p*I*. In the search, you can specify one or more terms matching those in the SWISS-PROT OS (species) or OC (classification) lines to limit the search to one organism, or a range of organisms. Thus, if you want

to investigate proteins exclusively from *S. cerevisiae*, you can specify "CEREVISIAE." This is better than specifying "YEAST," a word common to the classification of many yeasts, which includes not only proteins from *S. cerevisiae*, but also those from *Candida albicans* and *Schizosaccharomyces pombe*. The same applies for *Homo sapiens*, where "SAPIENS" will search only for human proteins, whereas "HUMAN" will include proteins from human viruses. If you would like to investigate proteins from a broader range of species, it is possible, for example, to specify a classification like "MAMMALIA," which will return all mammalian proteins within the specified pI and mol-wt region. Use of the word "ALL" will search all species in the database. If desired, searches can also be restricted through use of a SWISS-PROT keyword, such as "plasmid" or "AIDS." A document containing a full list of all SWISS-PROT keywords can be found at the URL address of: http://www.expasy.ch/txt/ keywlist.txt. Finally, select the "Start TagIdent" button to submit the request to ExPASy. Results will be sent in a few minutes to your e-mail address. A typical output is shown in **Fig. 4A**.

3.4.2. Use of TagIdent to Identify Proteins from a 2-D Gel

TagIdent can identify proteins by matching sequence tags against proteins in SWISS-PROT from one or more species within a specified pI and mol-wt range (*see* **Note 10**). To use TagIdent for identification purposes, first specify the pI and molecular weight of the protein of interest as estimated from the 2-D gel. Then specify error margins that reflect the known accuracy of these estimates. (*See* Wilkins et al. *[5]* for an example of how pI and mol-wt ranges can be defined.) The species and any keyword in the database to match against should then be specified (see **Subheading 3.4.1.**), the "Tagging" option selected by clicking in the small box, and the sequence tag entered in single amino acid code in the "Tag" text box. Note that the sequence tag can contain one or more "Xs" to represent if any unknown amino acid is unknown. Finally, you should specify the source of your protein sequence (N-, C-terminal, or internal), such that the program can show the protein area of interest in the search results. Thus, for example, if you have generated an N-terminal protein sequence tag by Edman degradation, you should request the program to show predicted protein N-termini. Finally, submit the search to the ExPASy server by selecting the "Start TagIdent" button. Results will be sent in a few minutes to your e-mail address.

3.4.3. Interpretation of TagIdent Results for Protein Identification

Accurate identification of proteins with sequence tags relies on all proteins from an organism being in sequence databases. In this manner, if only one protein within a given pI and mol-wt range is found to contain a certain N-,

C-terminal or internal sequence tag, one can be confident that there is no other, as yet undescribed protein that could otherwise match the tag. In fully sequenced organisms, the procedure is thus self-checking. Because of this, the TagIdent approach is very useful for organisms, such as *Haemophilus influenzae*, *Mycoplasma genitalium*, *Methanococcus jannaschi*, *E. coli*, and even the eukaryote *S. cerevisiae*, whose genomes are known. A TagIdent output for a protein from *E. coli* is shown in **Fig. 4B**, and illustrates the specificity of the approach. Caution is advised where using TagIdent for the identification of proteins from poorly molecularly defined organisms or organisms that contain large protein numbers (e.g., human) (*see* **Note 11**). A four-amino acid sequence tag (of 160,000 different combinations) can be unique in microorganisms that have a total protein count of 500–6000, but less useful in, for example, humans, that have 50,000–100,000 different proteins. If protein identification results with TagIdent show more than one protein carrying the sequence tag in the expected region, the same sequence tag, p*I*, and mol-wt data can be used in conjunction with protein AA composition for identification with the AACompIdent tool (*see* **Subheading 3.5.2.**).

3.5. AACompIdent Tool

The AACompIdent tool *(5)* can identify proteins by their amino acid (AA) composition. The program matches the percent empirically measured AA composition of an unknown protein against the theoretical percent AA compositions of proteins in the SWISS-PROT/TrEMBL database. A score, which represents the degree of difference between the composition of the unknown protein and a protein in the database, is calculated for each database entry by the sum of the squared difference between the percent AA composition for all amino acids of the unknown protein and the database entry. All proteins in the database are then ranked according to their score, from lowest (best match) to highest (worst match). Estimated protein p*I* and molecular weight, as well as species of interest and keyword can also be used in the identification procedure (*see* **Note 9**).

3.5.1. Basic Use of the AACompIdent Tool

After selecting the AACompIdent tool from the Tools page, you must first choose the relevant AA constellation to use in matching. For AA compositions determined by standard methods, use Constellation 2. This constellation is for 16 AAs (Asx, Glx, Ser, His, Gly, Thr, Ala, Pro, Tyr, Arg, Val, Met, Ile, Leu, Phe, Lys), does not consider Cys or Trp, and calculates Asn and Asp together as Asx, and Glu and Gln together as Glx (*see* **Note 12**). You should then specify the e-mail address to which the results should be sent, and then scroll down to the "Unknown Protein" field. Here you should specify a name for the search

```
A
--------------------
Query name: CH8055
Search performed with following values:
pI =        6.5          Mw =        9000
delta-pI =  0.25         delta-Mw =  1800
OS or OC = PROTOZOA
KW keyword = ALL
--------------------
8 proteins found
      UBIQ_ACACA  (P49634)
              pI: 6.56, MW: 8595.81
              UBIQUITIN.

      UBIQ_DICDI  (P08618)
              pI: 6.56, MW: 8537.73
              UBIQUITIN.

      UBIQ_EIMBO  (P46574)
              pI: 6.56, MW: 8550.75
              UBIQUITIN.

      UBIQ_EUPEU  (P23324)
              pI: 6.55, MW: 8581.77
              UBIQUITIN.

      UBIQ_LEIMA  (Q05550)
              pI: 6.57, MW: 8548.78
              UBIQUITIN.

      UBIQ_TETPY  (P20685)
              pI: 6.56, MW: 8524.72
              UBIQUITIN.

      UBIQ_TRYBB  (P15174)
              pI: 6.57, MW: 8506.74
              UBIQUITIN.

      UBIQ_TRYCR  (P08565)
              pI: 6.56, MW: 8508.72
              UBIQUITIN.
--------------------
```

Fig. 4. **(A)** Output of the TagIdent tool where it was used to list all proteins in a specified p*I* and mol-wt region for one or more species. This approach is useful to show proteins in the database that may correspond to the identity of a protein on a 2-D gel. Here we inquired regarding the possible identity of a protein of approximate mass 9000 and of p*I* 6.5 from a protozoan. The protein ubiquitin was found to be present in many protozoa in this p*I* and Mw region. Although ubiquitin was the only protein present in the output here, the identity of the protein as ubiquitin would need to be confirmed by analytical techniques. Note that this approach can also be used where the mass of an entire protein has been accurately determined by mass spectrometry. In such a case, the mass window used for searching can be quite small (e.g., mass ± 0.5%).

that will appear as the subject of the e-mail message, the protein p*I* and mol-wt estimated from the 2-D gel as well as error ranges that reflect the accuracy of these estimates. You should also specify a keyword, if appropriate, and one or more terms matching those in the SWISS-PROT OS (species) or OC (classifi-

B

```
Search performed with following values:
pI =        5.97           Mw =        45098
delta-pI =  0.50    delta-Mw =         9019
OS or OC =  ESCHERICHIA
KW keyword =        ALL
Sequence Tag =      MDQT
Display the N-terminal sequence.
Print only the sequences matching your tag MDQT.


--------------------
297 proteins found
---
Results with tagging: 1 found
The sequence tag itself is printed in lowercase.
---
 DHE4_ECOLI (P00370)
  NADP-SPECIFIC GLUTAMATE DEHYDROGENASE (EC 1.4.1.4) (NADP-GDH).
  pI: 5.98, MW: 48581.37
 1     mdqtYSLESFLNHVQKRDPNQTEFAQAVREVMTTLWPFLE...

--------------------
```

Fig. 4. **(B)** Output of the TagIdent tool where it was used for protein identification. A protein from an *E. coli* 2-D gel was uniquely identified by virtue of its N-terminal sequence tag, estimated p*I*, and mass. Although the program was requested here to display protein N-termini, it will show any protein that carries a specified tag in the "results with tagging" list, be it found at a protein N-terminus, C-terminus, or internally. Here the identification of the protein as DHE4_ECOLI is convincing not only because the tag is at the amino terminus, but because the tag was not found anywhere in the sequence of the other 297 proteins also within the specified p*I* and mol-wt window.

cation) lines to limit the search to one organism, or a range of organisms (*see* **Subheading 3.4.1.** and the SWISS-PROT list of species abbreviations at URL address http://www.expasy.ch/cgi-bin/speclist). Matching can also be done against all species in the database by specifying "ALL." Finally, specify the experimentally determined AA composition of the protein, with compositional data expressed as molar percent. If you have analyzed a calibration protein in parallel with unknowns as part of your AA analysis procedure, the composition of this protein can be used to compensate for error inherent to the AA analysis procedure (*see* **Note 13**). To do this, go to the "Calibration Protein" field, specify the SWISS-PROT ID name for the protein (e.g., ALBU_BOVIN for bovine serum albumin), and enter the experimentally determined AA composition of the protein, with data expressed as molar percent. Finally, select the "Search" button to submit the data to the ExPASy server. Results will be sent to your e-mail address.

3.5.2. Use of the AACompIdent Tool with Sequence Tags

Protein samples from 2-D gels can be submitted to Edman protein sequencing to create a "sequence tag" of three or four amino acids, after which the

same protein sample can be used for AA composition analysis *(6)*. This approach provides protein identification of higher confidence than identification by amino acid composition analysis alone. To use AA composition and sequence tag data together for protein identification, fill in the AACompIdent form as in **Subheading 3.5.1.**, but do not immediately submit it to ExPASy. Go to the bottom of the form, select the tagging option by clicking in the small box, and enter a protein sequence tag of up to six amino acids in single AA code into the "Tag" text field. Finally, specify if the sequence tag is N- or C-terminal, and select the "Search" button to submit the data to the ExPASy server. Results will be sent to your e-mail address.

3.5.3. Interpretation of AACompIdent Results

The output of AACompIdent contains three lists of proteins ranked according to their AA score (**Figs. 5A,B**). The first list contains is the results from of matching the AA composition of the query protein against all proteins from the species of interest, that have the specified keyword (if any), but without considering the specified p*I* and molecular weight. The second list shows the result of matching the AA composition of the query protein against all proteins from all species in SWISS-PROT that have the specified keyword, again without considering p*I* and molecular weight. The third list contains the results of matching the AA composition of the query protein only against the proteins from the species of interest that lie within the specified p*I* and mol-wt range (*see* **Note 8**) and that also have the appropriate keyword. The third list is the most powerful search. In all lists, a score of 0 indicates a perfect match between the query protein and a protein in the database, with larger scores indicating increasing difference.

We have found that a top-ranked protein is likely to represent a correct identification if it meets three conditions (**Fig. 5A**). First, the same protein, or type of protein, should appear at the top of the three lists. Second, the top-ranked protein in the third list should have a score <30 (indicating a "good fit" of the query protein with that database entry). Finally, the third list should show a large score difference between the top-ranked protein and the second-ranked protein (indicating a unique matching of the query protein with the top-ranked database entry). For proteins from *E. coli*, we have shown that a score difference greater than a factor of 2 gives high confidence that the top-ranked protein represents the correct identity *(5)*. If the top-ranking protein in the results does not meet these three conditions, the correct identity is often within the list of best-matching proteins (*see* **Note 14**). In such cases, the use of AACompIdent with a protein sequence tag can provide unambiguous identification owing to the high specificity of sequence tag data *(6)*. **Figure 5B** shows the result of protein identification by AA composition, p*I*, molecular weight, species and

sequence tag. Note that when the sequence tag option is selected, the AACompIdent output will show 40 amino acids of each protein's predicted N- or C-terminal sequence instead of its description, and show an asterisk to the left of a protein's rank if the protein carries the sequence tag anywhere in its sequence. If the tag is found in the displayed N- or C-terminal sequence, it will be shown in lower-case letters. We are confident that a protein from SWISS-PROT represents a correct identification if the query protein's empirically determined sequence tag of three amino acids or more is present at the expected N- or C-terminal position, and that this protein is ranked within the first 10 or so closest entries by amino acid composition.

3.6. AACompSim Tool

The AACompSim tool allows the theoretical AA composition of one protein in the SWISS-PROT database to be compared to proteins from one or all species in the database (*see* **Note 15**). This serves two main purposes. Firstly, to allow the simulation of matching undertaken for identification purposes with AACompIdent (*see* **Subheading 3.5.**), and second, it allows the detection of weak similarities between proteins by comparison of their compositions rather than sequences, as explored by Hobohm and Sander *(7)*.

3.6.1. Use of AACompSim

To use AACompSim, first select the constellation of amino acids you wish to work with (*see* **Note 16**). If you wish to simulate matching undertaken with empirical data, you should specify constellation 2. To match against the database for detecting protein similarities, you should use all 20 amino acids in constellation 0. Then specify an e-mail address to where the results can be sent, the SWISS-PROT identification name (e.g., IPIA_TOBAC) or accession number (e.g., Q03198) of the protein you would like to compare against the database, and the SWISS-PROT abbreviation for the species to match against (e.g., "SALTY" for *Salmonella typhimurium*). If desired, matching can be done against all species in the database by specifying "ALL." Finally, select the "Search" button to submit the match to ExPASy. AACompSim will return three lists of proteins, similar to those from AACompIdent. A sample output is shown in **Fig. 6**.

3.7. MultiIdent Tool

Proteins can be identified by virtue of their peptide masses alone, but frequently other data are needed to provide high confidence identification. The same is true for protein identification with AA composition. Following our earlier observations that high confidence protein identification can be achieved with a combination of peptide mass and AA composition data *(8,9)*, we have developed the protein identification tool MultiIdent. This tool uses parameters

```
SEARCH VALUES:
Constellation 2
Calibration protein: OVAL_CHICK ( P01012 )
Species searched: ESCHERICHIA
Keyword searched: ALL
Name given to unknown protein: coli147

SpotNb coli147
==============
pI:   5.70  Range: (  5.20,    6.20)
Mw:  34894  Range: ( 27915,   41873)

The closest SWISS-PROT entries (in terms of AA composition)
for the species ESCHERICHIA and the specified keyword:
Rank Score    Protein    (pI      Mw)  Description
=====================================================
   1     5 MDH_ECOLI     5.61    32337 MALATE DEHYDROGENASE (EC 1.1.1.37).
   2    30 YHDH_ECOLI    5.63    34724 HYPOTHETICAL 34.7 KD PROTEIN IN MREB
   3    31 K6P2_ECOLI    5.75    32388 6-PHOSPHOFRUCTOKINASE ISOZYME 2
   4    33 ALKH_ECOLI    5.57    22284 4-HYDROXY-2-OXOGLUTARATE ALDOLASE
   5    35 YEIN_ECOLI    5.37    32910 HYPOTHETICAL 32.9 KD PROTEIN IN NFO

The closest SWISS-PROT entries (in terms of AA composition)
for any species and the specified keyword:
Rank Score    Protein    (pI      Mw)  Description
=====================================================
   1     4 MDH_SALTY     6.02    32451 MALATE DEHYDROGENASE (EC 1.1.1.37).
   2     5 MDH_ECOLI     5.61    32337 MALATE DEHYDROGENASE (EC 1.1.1.37).
   3    12 MDH_HAEIN     5.86    32542 MALATE DEHYDROGENASE (EC 1.1.1.37).
   4    12 MDHM_EUCGU    5.64    33326 MALATE DEHYDROGENASE.
   5    13 PUR2_CHICK    7.51   106543 PHOSPHORIBOSYLAMINE--GLYCINE LIGASE

The closest SWISS-PROT entries having pI and Mw values in the specified
range for the species ESCHERICHIA and the specified keyword:
Rank Score    Protein    (pI      Mw)  Description
=====================================================
   1     5 MDH_ECOLI     5.61    32337 MALATE DEHYDROGENASE (EC 1.1.1.37).
   2    30 YHDH_ECOLI    5.63    34724 HYPOTHETICAL 34.7 KD PROTEIN IN MREB
   3    31 K6P2_ECOLI    5.75    32388 6-PHOSPHOFRUCTOKINASE ISOZYME 2
   4    35 YEIN_ECOLI    5.37    32910 HYPOTHETICAL 32.9 KD PROTEIN IN NFO
   5    36 SUCC_ECOLI    5.37    41392 SUCCINYL-COA SYNTHETASE BETA CHAIN
```

Fig. 5. (**A**) Output from the AACompIdent tool where a protein from an *E. coli* 2-D gel has been correctly identified by matching its amino acid composition, estimated p*I*, and mol wt against database entries for *E. coli*. Protein amino acid composition was determined according to Wilkins et al. *(5)*. The correct protein identity, malate dehydrogenase, was the top-ranked protein in the three lists, and showed a large score difference between the top- and second-ranked proteins in the third list (where p*I* and mol-wt windows are applied), and also in the first list. (*See text* for more details about the significance of score patterns for identification confidence.) In the second list, where the amino acid composition of the query protein was matched against all entries in the SWISS-PROT database without considering protein p*I* and molecular weight, malate dehydrogenase from four different species was ranked in the top four positions. This illustrates that protein amino acid composition is well-conserved across species boundaries. Note that the AACompIdent output has been shortened for this figure.

of protein species, estimated p*I* and molecular weight, keyword, AA composition, sequence tag, and peptide mass fingerprinting data to achieve protein identification (Wilkins et al., in preparation). Currently, the program works by first

```
-------------------------------------------
SEARCH VALUES:
Constellation 2
Calibration protein: ALBU_BOVIN ( P02769 )
Species searched: SACCHAROMYCES
Keyword searched: ALL
Name given to unknown protein: YEASTJI7
Tagging: YTT
The N-terminal sequence of the protein will be printed.
-------------------------------------------

SpotNb YEASTJI7
===============
pI:   5.25  Range: (  4.75,   5.75)
Mw:  32018  Range: ( 25614,  38422)

The closest SWISS-PROT entries (in terms of AA composition)
for the species SACCHAROMYCES and the specified keyword:
Rank Score    Protein    (pI      Mw)  N-terminal Sequence
==========================================================
*  1    19 IPYR_YEAST   5.36    32184 TyttRQIGAKNTLEYKVYIEKDGKPVSAFHDIPLYADKEN
   2    23 YCY4_YEAST   7.59    44982 MVSLFKRGKAPPLTKEGPTSKKPPNTAFRQQRLKAWQPIL
   3    30 YJF4_YEAST   5.61    54182 MSSESGKPIAKPIRKPGYTNPALKALGIPALRLPSRNWMI
*  4    30 SYV_YEAST    5.95   119994 SDLDNLPPVDPKTGEVIINPLKEDGSPKTPKEIEKEKKKA
   5    30 YJG0_YEAST   6.17    50082 MKQRFIRQFTNLMSTSRPKVVANKYFTSNTAKDVWSLTNE

The closest SWISS-PROT entries (in terms of AA composition)·
for any species and the specified keyword:
Rank Score    Protein    (pI      Mw)  N-terminal Sequence
==========================================================
*  1    19 IPYR_YEAST   5.36    32184 TyttRQIGAKNTLEYKVYIEKDGKPVSAFHDIPLYADKEN
   2    23 YCY4_YEAST   7.59    44982 MVSLFKRGKAPPLTKEGPTSKKPPNTAFRQQRLKAWQPIL
   3    24 MTMU_MYCSP   9.04    26933 MENKVTAYSIYNKKAKKNTKVNPLDEVFPQLPRKKYQVIY
   4    26 PUR7_CANMA   5.09    32941 MTSTNLEGTFPLIAKGKVRDIYQVDDNTLLFVATDRISAY
*  5    26 RAC1_DICDI   6.82    21573 MQAIKCVVVGDGAVGKTCLLISyttNAFPGEYIPTVFDNY

The closest SWISS-PROT entries having pI and Mw values in the specified
range for the species SACCHAROMYCES and the specified keyword:
Rank Score    Protein    (pI      Mw)  N-terminal Sequence
==========================================================
*  1    19 IPYR_YEAST   5.36    32184 TyttRQIGAKNTLEYKVYIEKDGKPVSAFHDIPLYADKEN
   2    36 MT16_YEAST   5.62    29882 MKTYHLNNDIIVTQEQLDHWNEQLIKLETPQEIIAWSIVT
   3    38 YNK7_YEAST   5.26    25981 MAPTISKRIKTLSVSRPIIYGNTAKKMGSVKPPNAPAEHT
*  4    44 MM21_YEAST   5.02    30340 MALNDNPIPKSVPLHPKSGKYFHNLHARDLSNIYQQCYKQ
   5    45 YGF3_YEAST   5.01    27250 MQTPSENTNAKSDSLDEPGAYLIEENVALPKDIFHSYLSY
-------------------------------------------
```

Fig. 5. **(B)** Output from the AACompIdent tool where a protein from an *S. cerevisiae* 2-D gel was correctly identified by matching its amino acid composition, estimated p*I* and molecular weight and an N-terminal sequence tag of XYTT against database entries for *S. cerevisiae*. Protein sequence tag and amino acid composition were determined according to Wilkins et al. *(6)*. The correct protein identity, inorganic pyrophosphatase, was the top-ranked protein in all three lists and was the only protein to show the sequence tag at its predicted N-terminus. Note that when the sequence tag is found anywhere in the protein, it is indicated by an asterisk to the left of the protein rank, and the tag is shown in lower case if found in the displayed sequence. The AACompIdent output was shortened for this figure.

generating a set of proteins in the database with AA compositions close to the unknown protein, as for AACompIdent (*see* **Subheading 3.5.**). Theoretical peptide masses from the proteins in this set are then matched with the peptide

```
-------------------------------------------
Protein IPIA_TOBAC
==================
pI:   8.80  Range: (  8.55,   9.05)
Mw:   7891  Range: (  6313,   9469)

The closest SWISS-PROT entries (in terms of AA composition)
for the species ALL:
Rank Score    Protein    (pI       Mw)  Description
===================================================
    1      0 IPIA_TOBAC   8.80     7891 PROTEINASE INHIBITOR I-A.
    2     55 IPIB_TOBAC   9.18     7646 PROTEINASE INHIBITOR I-B.
    3     59 IER1_LYCES   8.74     7914 ETHYLENE-RESPONSIVE PROTEINASE INHIB
    4     60 ICID_SOLTU   8.76     8052 WOUND-INDUCED PROTEINASE INHIBITOR I.
    5     61 POL_SIVCZ    9.55    20169 PROTEASE.

The closest SWISS-PROT entries (in terms of AA composition)
for any species:
Rank Score    Protein    (pI       Mw)  Description
===================================================
    1      0 IPIA_TOBAC   8.80     7891 PROTEINASE INHIBITOR I-A.
    2     55 IPIB_TOBAC   9.18     7646 PROTEINASE INHIBITOR I-B.
    3     59 IER1_LYCES   8.74     7914 ETHYLENE-RESPONSIVE PROTEINASE INHIB
    4     60 ICID_SOLTU   8.76     8052 WOUND-INDUCED PROTEINASE INHIBITOR I.
    5     61 POL_SIVCZ    9.55    20169 PROTEASE.

The SWISS-PROT entries having pI and Mw values in the specified range
for the species ALL:
Rank Score    Protein    (pI       Mw)  Description
===================================================
    1      0 IPIA_TOBAC   8.80     7891 PROTEINASE INHIBITOR I-A.
    2     59 IER1_LYCES   8.74     7914 ETHYLENE-RESPONSIVE PROTEINASE INHIB
    3     60 ICID_SOLTU   8.76     8052 WOUND-INDUCED PROTEINASE INHIBITOR I.
    4     97 ICI1_LYCPE   8.73     8471 WOUND-INDUCED PROTEINASE INHIBITOR I.
    5    118 GRO2_RABIT   8.83     7877 GROWTH REGULATED PROTEIN HOMOLOG.
-------------------------------------------
```

Fig. 6. AACompSim output where the serine protease inhibitor IPIA_TOBAC was matched against proteins from ALL species in the SWISS-PROT database. Many other serine proteases of type potato chymotrypsin inhibitor I were found, including ICI1_LYCPE, which has only 49% sequence identity with IPIA_TOBAC. This illustrates that proteins that belong to a family have well-conserved amino acid compositions. Note how the proteins POL_SIVCZ and IPIB_TOBAC are not present in the third list because their p*I* and molecular weight do not fall within the default windows (p*I* ± 0.25, mass ± 20%). The AACompSim output was shortened for this figure.

masses of the unknown protein to find the number of peptides in common (number of "hits"). Three types of lists are produced in the results (**Fig. 7**): first, a list where proteins from the database are ranked according to their AA composition score (*see* **Subheading 3.5.**); second, a list where proteins are ranked according to the number of peptide hits they showed with the unknown protein; and third, a list that shows only proteins that were present in the both the above lists, where these proteins are ranked according to an integrated AA and peptide hit score. In all these lists, protein p*I*, molecular weight, species of origin, and SWISS-PROT keyword can be used as in AACompIdent to increase the specificity of searches. We believe the ability of this tool to use all these parameters makes it one of the most powerful protein identification programs available.

```
-------------------------------------------
Constellation 2 with peptide-mass fingerprinting
SEARCH VALUES:
Name given to unknown protein: DOG2
Calibration protein: ALBU_BOVIN ( P02769 )
Species searched: ALL
Max. number of proteins generated in list: 100
Tagging: PFGN
The N-terminal sequence of the protein will be printed.
The enzyme is Trypsin.
Cysteine in reduced form.
The peptide-masses range are 500 to 3000 and the tolerance is 3 Dalton.
-------------------------------------------


SpotNb DOG2
===========

pI:   7.20  Range: (  6.20,    8.20)
Mw:  44900  Range: ( 35920,  53880)

AMINO ACID COMPOSITION
The SWISS-PROT entries having pI and Mw values in the specified range
for the species ALL and the specified keyword:
Rank Score    Protein    (pI      Mw)  N-terminal Sequence
===========================================================
    1    16 GCST_YEAST   7.81    41161 GTMVPYAGYSMPVLYKGQTHIESHNWTRTNAGLFDVSHML
*   2    17 KCRM_CANFA   6.84    43022 pfgnTHNKFKLNYKPEEEYPDLTKHNNHMAKALTPEIYKK...
*   3    19 KCRM_MOUSE   6.58    43045 MpfgnTHNKFKLNYKPQEEYPDLSKHNNHMAKVLTPDLYN...
*   4    20 KCRM_RAT     6.58    43019 MpfgnTHNKFKLNYKSQEEYPDLSKHNNHMAKVLTPDLYN...
    5    20 KARG_PENJP   6.36    39991 VDAAVLEKLQAGFKKLEAATDCKSLLKKYLSKDIFDKLK...

PEPTIDE-MASS FINGERPRINTING
The closest SWISS-PROT entries for the species ALL and
keyword ALL, having pI and Mw values in the specified range:
The best-matching set of peptide-masses
Rank  Hits     Protein        Matched Peptide-masses
================================================================
*  1     8     KCRM_CANFA     2010.12 / 1787.02 / 1739.92 / 1658.85 / 1522.74 /
                              1508.58 / 1303.47 /  908.04
*  2     5     KCRM_RABIT     2010.12 / 1787.02 / 1508.71 / 1303.47 /  908.04
*  3     4     KCRM_MOUSE     1787.02 / 1508.71 / 1303.47 /  908.04
*  4     4       KCRM_RAT     1787.02 / 1508.71 / 1303.47 /  908.04
*  5     4     KCRM_HUMAN     1787.02 / 1508.71 / 1303.47 /  908.04


The best integrated scores
Rank  Int_score Protein        Modification
=========================================
*  1     2.1       KCRM_CANFA
*  2     4.0       KCRM_RABIT
*  3     4.7       KCRM_MOUSE
*  4     5.0         KCRM_RAT
*  5     5.0       KCRM_HUMAN
```

Fig. 7. Output from the MultiIdent tool where a protein from dog heart was unambiguously identified by matching its peptide mass data, amino acid composition, sequence tag, and estimated p*I* and molecular weight against database entries for ALL species in the SWISS-PROT database. Protein N-terminal sequence tag and amino acid composition were determined according to Wilkins et al. *(6)* and peptide mass fingerprinting was performed according to Wheeler et al. *(9)*. The correct identity of creatine kinase m chain (KCRM_CANFA) was not ranked at the top of the best-matched proteins by amino acid composition. However, the sequence tag of XPFGN was found at the predicted N-termini of creatine kinase from three species. Note that asterisks to the left of the protein rank show the presence of the sequence tag, and that the sequence tag is printed in lower case if found in the displayed protein sequence. Further evidence that the protein was creatine kinase was found in the lists generated by peptide mass fingerprinting and with integrated scores, where all top-five-ranked proteins in those lists were creatine kinases, and all carried the sequence tag. The MultiIdent output was shortened for this figure.

3.7.1. Use of the MultiIdent Tool

After selecting MultiIdent from the Tools page, you must first choose the constellation of amino acids you wish to work with (*see* **Note 3**). Then provide information including your e-mail address, details of unknown protein (name, p*I* and mol-wt estimations, amino acid composition, sequence tag data if available), species of interest for matching (*see* **Notes 9** and **14**), and SWISS-PROT keyword (if any). This should be done in essentially the same manner as for AACompIdent (*see* **Subheading 3.5.**). However, the layout of the MultiIdent form is slightly different. To include peptide masses for protein identification, first specify the size of the list to be created with the query protein's AA composition (e.g., 100). Then click in the small box next to the "Peptide Mass Fingerprinting" title in the program to enable this option, and enter the list of peptide masses into the text box with an accuracy of at least one decimal place, and specify if masses are monoisotopic or average. Note that spaces and newline characters are ignored, allowing data to be copied from other sources and pasted directly into the text box here. Then specify the enzyme used to create the peptides (e.g., trypsin), if the protein was reduced and alkylated with any reagent (e.g., iodoacetamide, iodoacetic acid, 4-vinylpyridene) before cleavage, if artifactual protein modifications, such as oxidation of methionine, or acrylamide adducts to cysteine are expected, and the mass tolerance to be used in matching with peptides. The mass tolerance should reflect the known accuracy of your mass spectrometer. Finally, specify which or all of the results lists you would like to see in the MultiIdent output (as described in **Subheading 3.7.**), and select the "Perform" button to submit the match to ExPASy. The results will be sent to you by e-mail.

3.7.2. Interpretation of Results

A shortened output from the MultiIdent tool is shown in **Fig. 7**. The list of closest SWISS-PROT entries in terms of protein AA composition is the same as for the AACompIdent output, and thus results and scores for proteins in this list can be interpreted as in **Subheading 3.5.3.** If sequence tags are used as part of a search strategy, the list of closest proteins in terms of protein AA composition will show the predicted protein N- or C-terminal sequence, and any sequence tags present will be highlighted in lower-case letters. Asterisks are also shown to the left of protein rank numbers to indicate that the sequence tag is present in the corresponding protein. These asterisks are used in the list of best matches by AA composition, as well as the lists of proteins generated by peptide mass fingerprinting and the integrated score.

The list of closest SWISS-PROT entries in terms of peptide hits is simply the list of proteins that have the most peptides in common with the query protein (**Fig. 7**). The "hits' are the number of peptides that match with a database

entry, and the peptide masses shown in the output are those from the database entry that match with those from the query protein. The top-ranked protein in this list will be the most likely identification of the protein. However, this will may probably not be so if matching has been done without very large mol-wt windows. In any case, use of sequence tags of even 3 or 4 amino acids with peptide mass data can greatly increase the confidence of a database entry representing a correct identification. Note that for this purpose, sequence tags data generated by tandem mass spectrometry (MS/MS) or by postsource decay MALDI-TOF techniques can be used in MultiIdent as well as tag data generated at protein N- or C-termini.

The list of proteins with best-integrated scores represents the most powerful form of matching (*see* **Note 17**). It can simultaneously consider the protein parameters of p*I*, molecular weight, AA composition, sequence tag, and peptide masses in order to rank proteins from the database for the species of interest (*see* **Notes 8** and **14**) with a given keyword. The integrated score is a measurement of difference between the query protein and a database entry, and is derived by dividing multiplying the AA analysis score by the number of peptide hits that were found for that protein. Accordingly, an integrated score of 0 represents a perfect match for a query protein, with larger scores representing increasing differences. We find that the integrated score is useful for defining confidence limits if it is not immediately apparent if a protein has been correctly identified, and are currently defining these limits through the examination of matching results obtained after analysis of many proteins from 2-D gels.

4. Notes

1. Protein p*I* is calculated using p*K* values of amino acids described in Bjellqvist et al. *(10,11)*, which were defined by examining polypeptide migration between pH 4.5 and 7.3 in an immobilized pH gradient gel environment with 9.2 and 9.8 *M* urea at 15 or 25°C. Prediction of protein p*I* for highly basic proteins is yet to be studied, and it is possible that current Compute pI/Mw predictions may not be adequate for this purpose. The buffer capacity of a protein will affect the accuracy of its predicted p*I*, with poor buffer capacity leading to greater error in prediction *(10,11)*. Because of this, p*I* predictions for small proteins can be problematic.

2. Protein molecular weight is calculated by the addition of average isotopic masses of amino acids in the protein and the average isotopic mass of one water molecule. This program does not account for the effects of posttranslational modifications. Thus, modified proteins on a 2-D gel may migrate to a position quite different from that predicted. Protein glycosylation in particular can affect protein migration in both p*I* and mol-wt dimensions. Note, however, that the "GET REGION ON 2D PAGE" function in SWISS-2DPAGE (accessed by selecting a "GET REGION ON 2D PAGE" hypertext link from a SWISS-PROT entry) uses the Compute pI/Mw algorithm to highlight the a region on a 2-D gel to where an

unmodified protein should run, and suggests a region where the modified protein might be found if it has modifications documented in the SWISS-PROT database.

3. The Compute pI/Mw tool can also be accessed directly from the bottom of any SWISS-PROT database entry. To get to a SWISS-PROT entry, queries can be done at URL address: http://www.expasy.ch/sprot/sprot-top.html

4. Detailed documentation and instructions for this program are provided through a hypertext link at the top of the user page.

5. The "Monoisotopic Mass" option is useful in the mass prediction of small peptides (<3000 Dalton), that can often be isotopically resolved on mass spectrometers. The $(M + H)^+$ option will calculate all peptide masses with an extra hydrogen atom to give values for singly charged peptides as found in electrospray and MALDI-TOF mass spectrometers.

6. The PeptideMass program does not predict potential posttranslational modifications in user-entered sequences and thus does not consider these in mass calculations. However, one can use the PROSITE database *(12)* (http://www.expasy.ch/sprot/prosite.html) or the Scan Prosite Tool (http://www.expasy.ch/sprot/scnpsite.html) to predict the presence of posttranslational modifications in a sequence. A list of modifications documented in the PROSITE database can be found at URL address: http://www.expasy.ch/cgi-bin/prosite-list.pl.

7. PeptideMass cannot supply the predicted masses of peptides or proteins carrying most *N*- or *O*-linked glycosylation or other complex modifications like glycan phosphatidylinositol anchors because of their unpredictable heterogeneity. However, the discrete *O*-GlcNAc and C-mannosylation modifications are considered.

8. Protein p*I* and molecular weight in TagIdent are calculated as described for Compute pI/Mw (*see* **Subheading 3.2.**).

9. Care must be taken in the use of p*I* and mol-wt estimates from 2-D gels as part of protein identification strategies. Windows around these estimates that are too narrow can exclude the correct identification from the list of candidate identifications. As a general rule, we use windows of pI ± 0.5 U for proteins from bacteria and yeast, and pI ± 1.0 U for mammalian proteins. We generally use a mol-wt window of ±20%, but for proteins larger than 60,000 molecular weight, a window of ±10% is sufficient owing to the more accurate estimations (in percentage terms) that can be made with higher mass proteins on gels. If proteins are thought to be highly posttranslationally modified, very large p*I* and/or mol-wt windows may be needed.

10. TagIdent is extremely useful for searching proteins in the database for the presence of sequence tags, since it can search in a species-specific manner and with p*I* and mol-wt parameters. It thus avoids the drawbacks of the widely used FASTA *(13)* and BLAST *(14)* programs, which are global searching tools that either cannot search with small sequence tags or return lists containing many irrelevant proteins.

11. If you specify parameters that generate an extremely large TagIdent output (>1 megabyte), only the first 1000 lines will be sent by e-mail. This is to avoid problems that can arise when large messages arrive at some e-mail sites.

12. If AA analyses yield unreliable data for one or more amino acids, such values can be ignored and matching undertaken only with "good" amino acids using the AACompIdent free constellation *(15)*. The free constellation also allows the user to modify the bias and weight for each AA, if desired.

13. When calibration proteins are used, AACompIdent compares the experimental composition of the protein against the theoretical composition in the SWISS-PROT database to create a factor set. This factor set is then applied to the experimental composition of the unknown protein before it is matched against the SWISS-PROT database. Use of calibration proteins can increase identification efficiency dramatically, and is advised wherever possible. Note, however, that calibration proteins should be electrophoretically prepared in the same manner as unknown proteins and subjected to AA analysis in parallel with unknown proteins. It is also essential that the complete sequence of calibration proteins be in the SWISS-PROT database, since calibration cannot be done if only a fragment of the calibration protein sequence is available.

14. Protein AA composition and molecular weight are highly conserved across species boundaries, and serve as useful parameters for cross-species protein identification *(8,16)*. Protein p*I* is, however, poorly conserved between species. Cross-species protein identification in AACompIdent can be done by specifying "ALL" for the species of interest or specifying the SWISS-PROT species code of a well-defined organism that is closely related to the species under study. It must be noted that high confidence cross-species protein identification usually requires peptide mass data or sequence as well as AA composition (*see* **Subheading 3.7.**).

15. AACompSim automatically uses the theoretical p*I* and molecular weight of the specified protein in the matching procedure. The p*I* and molecular weight are calculated as in Compute p*I*/Mw (*see* **Subheading 3.2.**).

16. Default windows of p*I* ± 0.25 and mol wt ± 20% are used by AACompSim in matching. However, matches undertaken without these windows are also included in the program output.

17. Although peptide masses for any protein type are not as well conserved across species boundaries as other parameters *(16)*, they can be used for cross-species protein identification in conjunction with, for example, AA composition *(8,9)*.

Acknowledgments

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54

Automated Protein Identification Using Microcolumn Liquid Chromatography-Tandem Mass Spectrometry

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1. Introduction

The sequencing of complete genomes is providing an enormous resource for understanding the biology of organisms *(1)*. A complete genome sequence, however, will still leave many unanswered questions about the basic biology of the organism. Which open reading frames are authentic genes? What genes express highly abundant proteins in the cell? How well do the observed physical properties of a protein agree with the predictions of the genomic sequence? Which proteins are covalently processed? Analysis of the proteins encoded in the genome or "the proteome" can answer many of these questions, and will help in understanding the physiology of the organism.

As elegantly illustrated in this book two-dimensional electrophoresis (2-DE) can separate 2–4000 proteins in a single analysis *(2,3)*. The separation is based on independent, orthogonal physical properties related to the isoelectric point and molecular mass of each protein. This technique has the power to separate a complex mixture of proteins into single spots on a gel. From the 2-DE gel, a protein's observed pI and M_r are precisely measured. In addition, the amount of protein in a 2-DE spot generally reflects its in vivo abundance. Although 2-DE is effective in displaying the proteome, correlating protein identity to the genomic sequence has not been straightforward. Edman sequencing is a slow process and lacks the required sensitivity. The method also requires unblocked amino-termini and so cannot be used for the identification of most 2-DE spots from eukaryotic cells *(4)*. Amino acid composition identification is more sen-

sitive and relatively fast, but reliable identification by this method requires other information, such as p*I*, M_r, N-terminal sequence, or peptide mass data *(5)*. Mass spectrometry has been used to determine the mass of peptides produced by site-specific proteolysis of proteins obtained from 2-DE spots *(6)*. These data form a peptide mass map that can be used to search protein databases to identify proteins that would produce the same set of peptide masses if digested with same protease *(7)*. Problems occur with this method when a 2-DE spot contains a mixture of proteins, contaminants are encountered, or the protein is heavily modified. These situations can lead to ambiguous or false identifications.

A second mass spectrometry method for identifying proteins involves tandem mass spectrometry (MS/MS). Tandem mass spectrometry separates a peptide ion from a mixture of ions for dissociation by an activation method, such as collision-induced dissociation. By inducing multiple low-energy collisions with a neutral, inert gas such as argon or helium, ions are activated to dissociate *(8,9)*. In a second step or mass analyzer, the *m/z* values of the fragments are separated and then detected. The fragmentation pattern of a peptide is diagnostic and predictive of the peptide's amino acid sequence. Although tandem mass spectra can be manually interpreted to determine an amino acid sequence, a computer approach has been developed that uses tandem mass spectra to search protein or translated nucleic acid databases to identify the amino acid sequence represented in the tandem mass spectrum *(10,11)*. A single tandem mass spectrum representing seven to eight amino acid residues is sufficient to identify a protein. If covalent modification of the peptide is suspected, the experimental tandem mass spectrum can be searched against a protein database considering covalent modifications to specific amino acid residues *(12)*. By coupling an autosampler to microcolumn liquid chromatography and to a tandem mass spectrometer, complex peptide mixtures can be separated and sequenced in an automated manner *(10,12)*. This is a powerful approach to analyze proteins isolated from 2-DE.

2. Materials
2.1. Equipment

1. HPLC, HP1100, Hewlett-Packard (Wilmington, DE).
2. Autosampler, FAMOS, LC Packings (San Francisco, CA).
3. Workstation computer, DecStation 3000/300, Digitial Equipment (Maynard, MA).
4. Electrospray ionization ion trap mass spectrometer, LCQ, Finnigan MAT, San Jose, CA.
5. Fused Silica Capillary, Polymicro Technologies, Tucson, AZ.
6. Underivatized silica particles, 5 μ, EM Separations, Gibbstown, NJ.
7. POROS 10 R2 packing material, 10 μ, PerSeptive Biosystems, Framingham, MA.

2.2. Reagents

1. HPLC buffer A: 0.5% acetic acid in distilled and deionized water. HPLC buffer B: 0.5% acetic acid in 80% acetonitrile, 20% distilled, and deionized water.
2. Glacial acetic acid.
3. Methanol HPLC grade.
4. Angiotensin I, Sigma (St. Louis, MO).

3. Methods

3.1. Automated Microcolumn HPLC and Tandem Mass Spectrometry

3.1.1. Column Packing

1. Micro-columns are made using the method of Kennedy and Jorgenson employing 100-μm ID fused-silica capillary tubing (13). A 30-cm piece of fused silica capillary (200 μm OD × 100 μm id) is rinsed with 2-propanol, dried, and the tip is tapped into a vial of underivatized silica to create a frit. A cool flame is then used to sinter the particles of silica to the walls of the column (*see* **Note 1**).
2. The columns are packed with PerSeptive Biosystems (Boston, MA) POROS 10 R2, a 10-μm reverse-phase packing material, to a length of 10–15 cm. Suspend approx 100 μg of packing material in 0.5 mL of methanol (*see* **Note 2**).
3. Pack column by placing packing slurry in a pressurized bomb and feeding capillary tubing in to the slurry. Pressurize the bomb to approx 400 psi, and verify that the column is packing under a low-power microscope.
4. Prep column before use by running a short gradient (0–80% B for 15 min) through the column, followed by loading 2 pmol of angiotensin I onto the column and eluting into the mass spectrometer.

3.1.2. Fluid Connections

1. An LC Packings FAMOS Model 900 autosampler is used for sample loading and control of the HPLC pumps and mass spectrometer. This autosampler allows small volume injections to be made with minimal losses.
2. A Hewlett Packard Series 1100 (Wilmington, DE) binary pump with in-line vacuum degasser is used for gradient formation (*see* **Note 3**).
3. Connect column to the 6-port valve of the FAMOS using a 50-μm id transfer line (FMS-TUB-50, LC Packings) and a zero dead volume union.
4. The pump outlet is connected to the 6-port valve through a splitting tee that reduces the flow from the pump from 150 μL/min to 0.8–1 μL/min using a length of restriction tubing made from fused silica. The restriction tubing is typically 365 μm OD × 50 μm id × 7 cm (*see* **Note 4**). The splitting tee should be placed as close to the 6-port valve as possible to reduce the amount of delay in the system.
5. Connect a 20-μL sample loop to the 6-port valve. These can be purchased from LC Packings or made using fused silica capillary tubing.
6. A sheath liquid (methanol/water [70:30] mixture containing 0.1% acetic acid) flows concentrically around the end of the column (into the LCQ) at a flow rate of 2–2.5 μL/min. The sheath is provided using a Fluid Delivery Module (FDM)

Table 1
Time and Flow Components of the HPLC System

Time	Flow	%B	Comment
0.1 min	500 μL/min	0%	
12 min	500 μL/min	0%	High flow to push sample onto column
16 min	150 μL/min	0%	
31 min	150 μL/min	80%	Gradient
32 min	150 μL/min	0%	
45 min	150 μL/min	0%	Flow to ensure gradient flows through column and column is re-equilibrated

from Michrom BioResources (Auburn, CA). The FDM is a pressurized reservoir system (*see* **Note 5**).

3.1.3. Electrical Connections

The FAMOS auxiliary outlet connections are used to control the start of the HPLC pump and of mass spectrometer acquisition. Contact closure connections are made between the autosampler and the pump and between the autosampler and the mass spectrometer. Cables are available from LC Packings and Hewlett Packard to make these connections. Cables can also be made using commonly available connectors.

3.1.4. Instrument Programming

1. The program used for the HP pump is shown in **Table 1**. This program consists of three parts. The purpose of the higher flow rate at the beginning of the program (**step 2** in **Table 1**) is to push the contents of the sample loop onto the column, so that the sample loop may be taken out of the flow path. Taking the sample loop out of the flow path reduces the amount of dead volume in the system allowing for faster LC runs. The second step is a linear gradient from 0 to 80% buffer B in 15 min. This gradient may be modified for individual needs. The third step is a 13-min push with 100% buffer A to move the gradient through the column and re-equilibrate the column (*see* **Note 6**).
2. The programming of the FAMOS autosampler can be accomplished in two ways. The easiest method is to use one of the preprogrammed injection methods and use a time-base method to initiate the contact closures. The μL injection method is the best choice, since it loads only as much sample as will be injected onto the column. The major disadvantage of this is that the sample loop cannot be taken off-line after the sample is pushed onto the column. Having the sample loop in the gradient flow path increases the analysis time because of the extra 20 μL of tubing the gradient must pass through before reaching the column. The second method is to write a user program. A user program is used on the FAMOS

autosampler in which each function of the autosampler is programmed step-by-step. This program is shown in **Table 2** with associated comments. The major steps in the program are the following: flush tubing with transport liquid (0.5% acetic acid in water) (steps 1–5), load 0.5–6.5 μL of sample into the sample loop (steps 6–8), load a plug of transport liquid into sample loop (steps 9–11), trigger start of HPLC program and mass spectrometer acquisition (steps 12–13), reset autosampler and wait for sample to be pushed out of sample loop onto column (steps 14–17), flush autosampler syringe (steps 18–22), and wait for HPLC program and mass spectrometer acquisition to finish (step 23).

3. Tandem mass spectra are acquired on the Finnigan MAT LCQ (San Jose, CA) electrospray ionization ion trap mass spectrometer through an instrument control algorithm preprogrammed by the instrument manufacturer. A unit mass resolution scan over an *m/z* range of 550–1200 is acquired. If an ion is present in the scan above, an ion abundance threshold of 200,000–1,000,000 counts (depending on the background noise present prior to beginning the autosampler run), then an MS/MS scan is initiated on the ion. The MS/MS scan range is set by assuming the charge state is +2. This does not prevent the acquisition of tandem mass spectra for peptides with a +3 charge state. A precursor ion isolation width of 3 Dalton is used to select precursor ions. A default collision energy of 35% is used to fragment the peptide for the MS/MS scan. The number of microscans is set at 5 with the maximum ion inject time set at 500 ms. The LCQ is run using the Sample List feature of the software.

3.2. Database Searching with Tandem Mass Spectra

1. Searches were performed on a DECstation 3000/300 Alpha workstation (Maynard, MA). Methods for data reduction, preliminary scoring, and crosscorrelation analysis used in the computer algorithm have been described in detail elsewhere *(10–12)*.

2. Databases can be obtained over the Internet using anonymous ftp to the National Center for Biotechnology Information (NCBI) (ftp://ncbi.nlm.nih.gov). Databases such as the GenBank database of nucleotide sequences, the OWL nonredundant protein database, Genpept protein database, the SWISS-PROT protein database, and dbEST—the collection of expressed sequence tag sequences—can be obtained from the NCBI site. The complete *Saccharomyces cerevisiae* sequence can be obtained from Stanford Genomic Resources (http://genome-www.stanford.edu). The *Haemophilus influenzae* database can be obtained from The Institute for Genome Research (TIGR) at (http://tigr.org).

3. Search parameters can be set in the sequest.params parameters file. This includes the database to be searched, whether it is a nucleotide or protein database, and whether to perform calculations using average or monoisotopic masses. The set of sequence ions (types a, b, c, d, v, w, x, y, and z) to be considered can also be selected as well as the relative abundance values to be used during the theoretical reconstruction of the tandem mass spectra. The masses of amino acids to consider for modified peptides can be input. The mass tolerance for fragment ions and for the peptide mass can be set. Enzyme or chemical cleavage specificity can

Table 2
User Program Used to Control the Autosampler, the HPLC,
and Data Acquisition on the Mass Spectrometer

Step	Comment
1 Injector valve position: INJECT	
2 Syringe valve position: NEEDLE	
3 Compressor: ON	
4 Aspirate 6.0 µL reag-A Speed: 2 H:02 mm	This volume is not loaded onto the column; it flushes the injector tubing
5 Wait: 5 s	
6 Injector valve position: LOAD	
7 Aspirate 6.5 µL Sample Speed: 2 H:00 mm	Vol. is variable between 0.5 and 6.5 µL
8 Wait: 5 s	
9 Aspirate 6.0 µL reag-A Speed: 2 H:02 mm	
10 Wait: 5 s	
11 Injector valve position: INJECT	
12 Auxiliary port 2 ON	Aux 2 starts LCQ, could use other Aux lines
13 Auxiliary port 4 ON	Aux 4 starts HP Pump, could use other Aux lines
14 Compressor: OFF	
15 Wait: 16 min	Allows sample to be pushed onto column before sample loop is taken off-line
16 Auxiliary port 2 OFF	
17 Auxiliary port 4 OFF	
18 Injector valve position: LOAD	
19 Syringe valve position: WASTE	
20 Unload Syringe Volume: 18.5 µL Speed: 3	Vol. = 12 µL + sample vol. from step 7
21 Syringe Valve Position: NEEDLE	
22 Needle-Wash Volume of 50 µL	
23 Wait: 30 min	Allows gradient to flow onto column and column to be re-equilibrated
24 End of User-Program	Automatically placed

be entered from the following list: no enzyme, trypsin, chymotrypsin, clostripain, cyanogen bromide, iodoso benzoate, proline endopeptidase, *Staphylococcus aureus* V8 protease, lysine endoproteinase, arginine endoproteinase, aspN-endoproteinase, and elastase.

4. The tandem mass spectrometry data file is then processed in two ways. First the precursor ion is removed and the remaining ions are normalized. All but the top 200 most abundant ions are then removed from the search file. This processed spectrum is used to search the database. To perform the crosscorrelation analysis described below, a second file from the tandem mass spectrum is created. The precursor ion is removed, and the spectrum is divided into 10 equal regions. Within each region, the ions are normalized to the most abundant ion. The molecular weight of the peptide in the tandem mass spectrum is calculated directly from the precursor ion. The program can differentiate a +1 from a +2 charge state for the peptide ion. If the ion is determined to be of a charge state >1 the program will assume it is +2 in the mol-wt calculation.

5. A search of the database involves scanning each entry to find linear combinations of amino acids, proceeding from the N- to the C-terminus, that are within some tolerance of the mass of the peptide represented by the tandem mass spectrum. Sequence selection can also be guided by the cleavage specificity of the protease used to create the peptide, including consideration of incompletely digested peptides from either side of the primary sites, or it can be performed with no assumptions about how the peptide was created. If a nucleotide database is searched, the nucleotide sequences are translated "on the fly" to protein sequences in six reading frames *(11)*. Chemical modifications can be considered by changing the amino acid mass used to calculate the masses of the peptides. The modified amino acid is then considered at every occurrence in the sequence *(12)*.

6. Once an amino acid sequence is within the defined mass tolerance, a preliminary evaluation is performed *(10)*. First, the number (n_i) of predicted fragment ions that match ions observed in the spectrum within the fragment ion mass tolerance and their abundances (i_m) are summed. If an ion series is continuous, that is, if consecutive sequence ions are present, then a component of the score, β, is incremented. A sequence that matches a continuous set of ions is weighted more heavily than one that randomly matches a few sequence ions. If an immonium ion is present in the spectrum, then the associated amino acid must be present in the sequence under consideration or an additional component of the score, ρ, is increased or decreased correspondingly. The total number of predicted sequence ions is also noted (n_t). A score is calculated for each amino acid sequence by using the following relationship in Eq. 1:

$$S_p = (\sum i_m) * n_i * (1 + \beta) * (1 + \rho)/n_t \tag{1}$$

7. Each of the top 500 scoring sequences are subjected to a crosscorrelation analysis. This is performed by reconstructing a model tandem mass spectrum for each of the amino acid sequences in the list of 500 and comparing each one to the processed experimental tandem mass spectrum (**step 4**) by using a crosscorrelation function. The crosscorrelation function is a very sensitive signal processing method used to compare the coherence of two signals *(14)*. This is performed, in effect, by translating one signal across another. If two signals are the same or very similar, the correlation function should maximize when there is
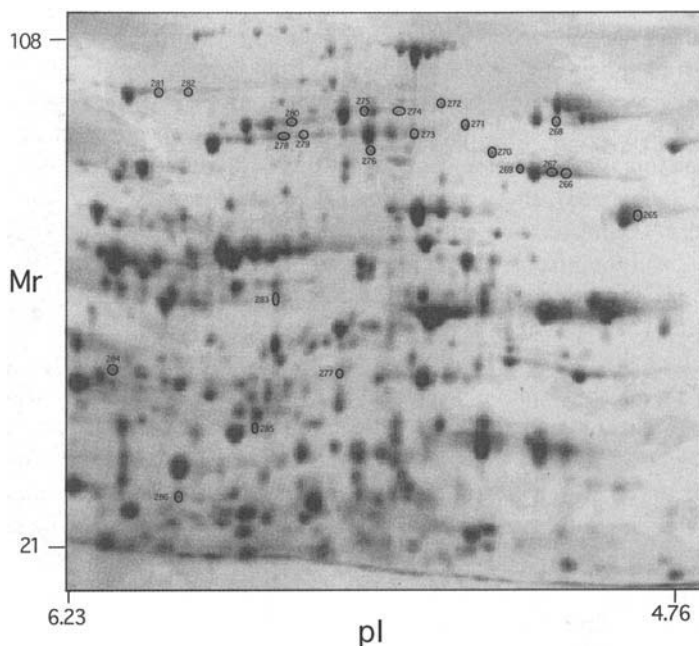
Fig. 1. Resolution of most *H. influenzae* proteins. 2-DE separation of the total-cell extract transferred to PVDF and Coomassie-stained. The first dimension used a linear pH 4.0–7.0 IPG strip gel, and the second-dimension used a 10% SDS-PAGE. The numbers are the 2-DE spot identification numbers.

no offset between the signals. A crosscorrelation score is computed for each of the 500 amino acid sequences. The values are ranked (Xcorr) and normalized (C_n).

3.3. Review of Search Results

1. Shown in **Fig. 1** is a two-dimensional gel electrophoretic separation of the total protein lysate from *H. influenzae*. Spots corresponding to 30 different proteins were removed, digested with trypsin as previously described *(15)*, and the proteins identified in the automated system. In 22 analyses, 19 proteins were identified (**Table 3**). Three identifications failed because no peptide tandem mass spectra were recorded with sufficient signal-to-noise to identify the proteins. The analysis of spot 285 identified two different proteins, methionyl-tRNA formyltransferase (HI0623) and malate dehydrogenase (HI1210).
2. Shown in **Fig. 2** is a summary of the output for a search with LC/MS/MS data. In the first column, the file name and the inclusive scan numbers for the tandem mass spectra used for the search are shown. This entry in the summary is linked to results for the individual searches (**Fig. 3**). Access to the individual search data allows the inspection of search results to look for sequence similarity between sequences.
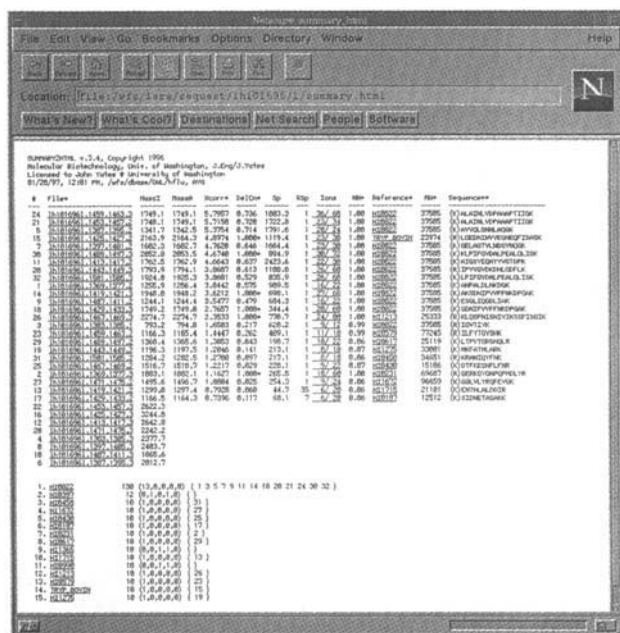
Fig. 2. Shown is the summary output for the search of the *H. influenzae* database using the tandem mass spectra obtained from LC/MS/MS analysis of a digested mixture of proteins.

3. In the second and third column, the peptide mass value calculated from the tandem mass spectrum (MassI) and the database sequence (MassA) are shown, respectively. The molecular weight of the peptide to be input into the search program is calculated automatically from the tandem mass spectrum.

4. In the columns marked Xcorr, DelCn, and Sp are recorded search scores. The values under Xcorr are the values obtained from the crosscorrelation analysis. The larger the value, the more similar the model tandem mass spectrum representing the database amino acid sequence is to the experimental tandem mass spectrum. The DelCn value is the difference between the normalized Xcorr value for the first and second search result. Again the larger the difference, the more dissimilar the first and second answers will be. The Sp scoring value is the preliminary scoring value as discussed above. The RSp value is the rank of the sequence on the basis of the preliminary score. A tandem mass spectrum with good signal-to-noise will generally rank first by the Sp value.

5. Under the column headings Ions, NN, and reference are the ratio of matched ions/predicted ions, a neural network evaluation of the scores for a search, and sequence reference number. The values in the Ions column are linked to a program that recreates the tandem mass spectrum, and identifies and marks the fragment ions in the spectrum for easy visualization (**Fig. 4**). A neural network has

Table 3
Proteins Identified Using the Automated Protein Identification System

Spot no. HI no.	Functional name	Peptides id (% protein)	p*I* (% diff.)	M_r, Dalton, (% diff.)
265 0543	Heat-shock protein	5 (17)	4.91 (–0.4)	59200 (–3)
266				
267				
268 1232	Dihydrolipoamide acetyltransferase	4 (12)	5.11 (1)	74100 (–25)
269				
270 0729	Prolyl-tRNA synthetase	6 (15)	5.27 (0.3)	68800 (–8)
271 0864	Hypothetical protein	5 (10)	5.34 (–3)	73400 (–7)
272 0924	Glycyl-tRNA synthetase β-chain	2 (4)	5.40 (–2)	77600 (–3)
273 0835	Fumarate reductase, flavoprotein subunit	4 (10)	5.48 (4)	7200 (–9)
274 1203	Hypothetical protein	3 (5)	5.52 (3)	76000 (0.6)
275 1203	Hypothetical protein	7 (15)	5.61 (2)	76000 (0.5)
276				
277 0564	Asparagine synthetase A	3 (12)	5.68 (–2)	40000 (–7)
278 0583	2',3'-cyclic-nucleotide 2'-phosphodiesteriase	3 (8)	5.84 (6)	71500 (2)
279 0835	Fumarate reductase, flavoprotein subunit	3 (7)	5.78 (–1)	71600 (–9)
280 1023	Transketolase 1	2 (4)	5.82 (–3)	74100 (–2)
281 1245	Malic acid enzyme	6 (13)	6.20 (–1)	79900 (2)
282 1245	Malic Acid enzyme	2 (3)	6.11 (0.4)	79900 (2)
283 1633	Adenylosuccinate synthetase	3 (10)	5.85 (–1)	48500 (–2)
284 1547	DAHP synthetase (phenylalanine repressible)	6 (25)	6.34 (–3)	40400 (–1)
285.1 0623	Methionyl-tRNA formyltransferase	1 (4)	5.91 (10)	34500 (1)
285.2 1210	Malate dehydrogenase	5 (29)	5.91 (–1)	34500 (–6)
286 0141	Glucosamine-6-phosphate deaminase protein	2 (9)	6.14 (–3)	28200 (7)

[a]The spot no. relates to the location of the spot taken from the 2-DGE shown in **Fig. 1**. The HI no. is the *H. influenzae* accession number. The functional name is the name ascribed by sequence similarity. The next column shows the number of peptides that matched to the protein as well as the percentage of the protein sequence identified. The next two columns show the observed p*I*s and M_r for the identified proteins, and the % difference from the calculated value is shown. A blank entry next to a spot no. indicates that no MS/MS spectra of sufficient quality were obtained to make an identification.

Fig. 3. Following the link from the File designations displayed in the first column (**Fig. 2**), the information for individual searches of tandem mass spectra can be found. This is useful to determine if a close score is the result of identifying two closely related sequences.

been trained to evaluate the scoring output for a search. A high score (e.g., 1.00) indicates a good match, and a low score (e.g., 0.067) indicates the sequence represented by the tandem mass spectrum is not present in the database. The entries in the Reference column are linked to sequence databases (**Fig. 5**) to display the sequence of the protein identified, and marked within the sequence is the specific region represented by the tandem mass spectrum. Direct access to the SWISS-PROT database entry for this particular protein sequence can be obtained through the link on this page. A Blast search with the sequence identified is also possible directly from this page.

6. The last two columns show the calculated molecular weight of the protein in which the sequence was found and the peptide sequence represented in the tandem mass spectrum.

7. At the bottom of **Fig. 2** is a compilation of the proteins identified in the LC/MS/ MS analysis. A rudimentary scoring system is used based on the multiplication of a first place answer by 10, a second place answer by 8, and so on. Each reference number is linked to summary of the matches within the protein sequence. Shown in **Fig. 6** is the summary for HI0822 D-galactose binding periplasmic protein. The regions of the protein identified are shown in a different color. A calculation of the sequence coverage is also included.
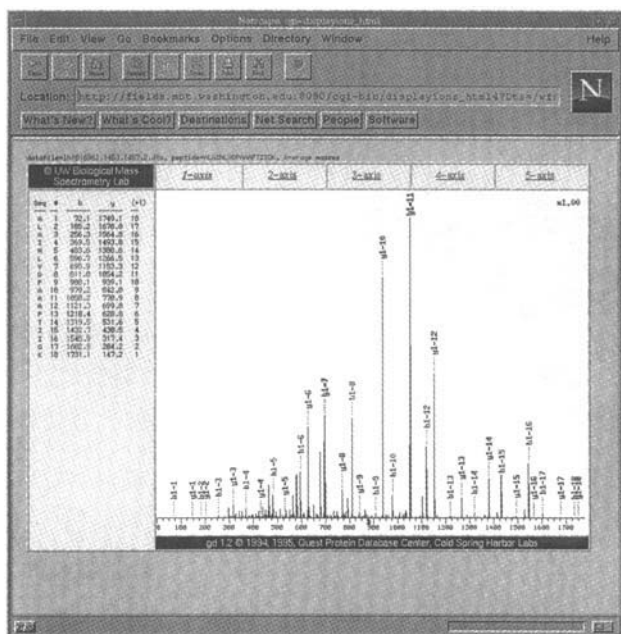
Fig. 4. Following the link from the Ions column shown in **Fig. 2**, the tandem mass spectrum used in the search can be found. The predicted fragment ions for the top-ranking sequence are shown to the left of the spectrum. The position of the fragment ions within the spectrum are indicated with the type of sequence ion. This information is displayed in different colors: red and blue for N-terminally and C-terminally related fragment ions, respectively.

3.4. Confirmation of Protein Identification

In general, the matches returned by SEQUEST are manually confirmed. The following are some guidelines for confirming the match:

1. Choose the candidate matches to be checked based on Xcorr, deltaCn, charge state, and whether there are multiple peptides from the same protein.
   a. For general purposes, use an Xcorr cutoff of 3 for all spectra. However, if the deltaCn is above 0.1, look at spectra with Xcorr above 2.8. These values may depend somewhat on instrument type and peptide origin. The above values work well for peptides generated by tryptic digestion and MS/MS spectra obtained using a TSQ700 or LCQ.
   b. These criteria change if the match is one of several from a single protein. Then look at all of the matches independent of score.
   c. In general, peptides with +3 charge state have to have an Xcorr of 3.5 and deltaCn above 0.1 before they are likely to be a confirmable match. Even then, some are questionable, especially with Xcorr between 3.5 and 4.0.
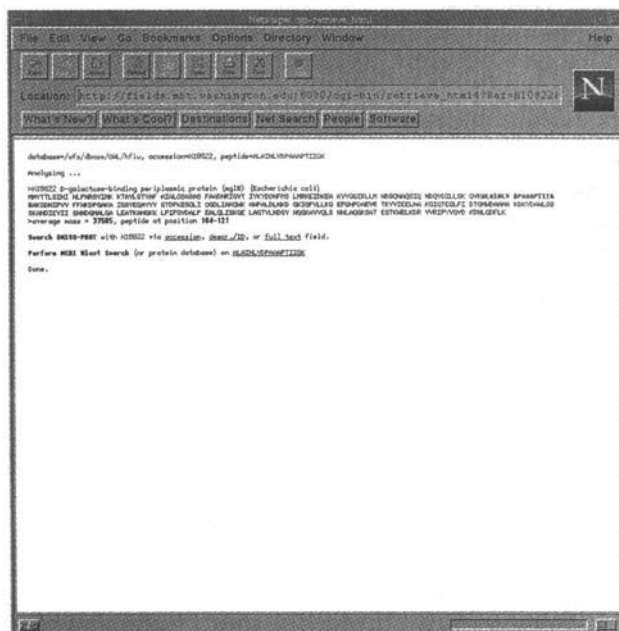
Fig. 5. Following the link from the Reference column shown in **Fig. 2**, the full sequence of the protein identified can be viewed. Within the full sequence, the amino acids represented in the tandem mass spectrum are shown with a different color. For this search result, the sequence SDLFNINAGIVR is highlighted in red.

2. Check spectra for quality. This is quite subjective. The more distinguishable the ion series peaks are from the background, the higher the quality of the spectra.
3. Check ion list for continuity in the fragment ion series. There should be continuous strings of matching ions as opposed to isolated matched ions. This is especially true for peptides with +3 charge state. Many times in good +3 spectra there will be a string of +2 y-ions covering the high-mass range and a string of +1 y-ions covering the low-mass range. Obviously, not all the predicted ions will be present, but a significant number should appear in a confirmed match.
4. Check for proline peaks.
    a. The y-ions that correspond to loss of a proline are often intense peaks. There may also be a corresponding +2 peak. These will normally be present for prolines occurring closer to the ends of peptides.
    b. If there are multiple prolines present, you may also see internal fragment ions that correspond to the mass of the peptide between the two prolines.
    c. Glycine peaks also tend to be relatively intense.
5. Check unidentified, intense peaks. Sometimes there may be a peak that corresponds to the loss of one or two amino acids from one end of the peptide. Also, be aware of the possibility of peaks resulting from the loss of water from a fragment

Fig. 6. Following the link from the information shown at the bottom of **Fig. 2**, a visual summary of all the tandem mass spectra matching to a particular protein sequence can be shown. A synopsis of the amount of information obtained is shown as well as links to the SWISS-PROT database to obtain sequence annotation information.

　　ion. The presence of several unexplainable, intense peaks is cause to question the validity of a match.
  6. Matches that may not be acceptable alone can be correct if they identify a protein for which there are other matches in the same LC/MS/MS run.

　　See **refs.** *16* and *17* for more detailed information on interpreting MS/MS spectra of peptides.

4. Notes
  1. It is important that the end of the capillary not have a jagged edge (as seen under a low-power microscope) or it will be difficult to get a frit to stay.
  2. Other packing materials may be used. The packing material will have a tendency to settle out; shake the tube to resuspend occasionally.
  3. Any HPLC pump that will run multiple programs started by a contact closure signal can be used. Dual-syringe pumps are limited in the volume that can be delivered per run. This can limit the length of gradient that can be used.
  4. The exact length and id of the restriction tubing will depend on the individual setup. The flow rate going into the mass spectrometer should be measured at the

end of the electrospray needle and the length of restriction tubing adjusted accordingly. Too high a flow rate through the column means less restriction is needed.

5. The sheath can be provided using any system that can provide a steady flow of 2–2.5 μL/min for a time period of 10–20 h without user intervention. The FDM is the best solution we have found.

6. The HPLC program shown is that used in our laboratory. Depending on the length of the flow path setup, some of the times will need to be changed. The critical times are those required to push the sample out of the sample loop before the loop is taken off-line and the length of time the system must flow after the pump has delivered the gradient. This last time must be long enough to get the gradient through the column as well as re-equilibrate the column with starting buffer before the next sample is loaded into the sample loop.

7. The angiotensin I is used to prep the column as well as verify that the system is functioning properly. An intense peak should be observed in the mass spectrometer corresponding to the angiotensin eluting. The +3 peak is typically seen at *m/z* 433.3.

8. The lifetime of a column will vary depending on the samples. The main concern is plugging a column with a precipitate or particulate from the sample. It is important to centrifuge samples before loading. Thirty 2-D gel spot digests have been run on a single column with no problems. However, be conservative and start with a new microcolumn before an overnight run to reduce the chances of a column clogging and the samples being lost.

9. When starting the sample list on the LCQ, select the Contact Closure button in Start Options. This causes the LCQ to wait until it receives a contact closure signal from the FAMOS before beginning the acquisition.

10. The SEQUEST software can provide highly accurate protein identifications. The highly specific information represented in a tandem mass spectrum allows proteins present in mixtures to be identified, since each tandem mass spectrum is a specific address to a protein in the same manner that an amino acid sequence can be highly specific for a protein. Generally several or more spectra are obtained for each protein, but a protein can be identified on the basis of one tandem mass spectrum if the following criteria are met: the amino acid sequence represented by the tandem mass spectrum is at least seven amino acids in length, the tandem mass spectrum contains a sufficient number of sequence ions to allow validation of the identified sequence, and the amino acid sequence is unique to a single protein within the organism from which the protein was derived.

11. SEQUEST is ideal for creating a high-throughput, automated system for the identification of proteins from 2-D gels. By combining an autosampler with an HPLC, protein digests can be automatically injected, separated, and tandem mass spectra automatically acquired. Tandem mass spectra can be acquired through an instrument control program on the Finnigan LCQ ion trap mass spectrometer allowing unattended acquisition. Proteins present in simple mixtures can also be identified through the acquisition of tandem mass spectra and matching the spectra to their respective proteins. Database sequence errors can be tolerated by

adjustment of parameters, such as the ion series and mass tolerance to be used in a search.

12. All tandem mass spectra should be validated against the sequence matched in the database.

13. SEQUEST will match a tandem mass spectrum to a similar, mass conserved sequence if the correct sequence is not present in the database.

14. SEQUEST can match tandem mass spectra with relatively poor signal-to-noise to the correct sequence, but the spectrum should be of sufficient quality for validation or the match should be considered tentative. Multiple hits to the same protein sequence with poor-quality tandem mass spectra can be considered a valid identification.

Acknowledgments

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55

Peptide Sequencing of 2-DE Gel-Isolated Proteins by Nanoelectrospray Tandem Mass Spectrometry

Ole Nørregaard Jensen, Matthias Wilm, Andrej Shevchenko, and Matthias Mann

1. Introduction

Shortly after the introduction of electrospray as a viable ionization technique for large molecules *(1)*, electrospray tandem mass spectrometry (ES MS/MS) techniques, such as HPLC-ES MS/MS, were used successfully for peptide sequencing at picomole and subpicomole levels *(2–4)*. In LC-MS/MS, peptide sequence information is generated during the short time, 10–30 s, that a peptide elutes from the HPLC column run at a flow rate of 0.5–5 μL/min. This time frame rarely allows optimization of experimental parameters for MS/MS sequencing of individual peptides unless several LC-MS/MS experiments can be performed on a sample. With the introduction of the nanoelectrospray ion source *(5–7)*, the time constraint of tandem mass spectrometry has been removed, and peptide sequencing has been reliably extended to the femtomole level of gel-isolated protein.

The main characteristics of the nanoelectrospray ion source are low flow rate, high ionization efficiency, and extended measurement time with concomitant improvements in absolute sensitivity *(5,6)*. These features are crucial for peptide sequencing by tandem mass spectrometry. In our laboratory, nanoelectrospray tandem mass spectrometry is a reliable and robust technique for identification or sequencing of gel-isolated proteins available in sub-picomole amounts *(7–12)*. The very low flow rate of 10–25 nL/min of the nanoelectrospray source has required a change of sample preparation method from on-line HPLC separation to sequencing directly from desalted/concentrated peptide mixtures as will be explained below.

For nanoelectrospray tandem mass spectrometry and other mass spectrometric methods, it is important to choose the most suitable protease for generation of peptides from a protein. Trypsin has several advantages for the generation of peptides for tandem mass spectrometric sequencing. It is an aggressive and extremely specific protease that cleaves the C-terminal amide bond of lysine and arginine residues. Tryptic peptides typically have a mass between 800 and 2500 Dalton, and both the N-terminal amino group and the C-terminal residue (Lys or Arg) are basic, so they generate mainly doubly charged peptide ions in electrospray. Such ions predominantly fragment at the peptide amide bond to generate singly charged N-terminal (b-type) or C-terminal (y-type) ions *(13,14)* (*see* **Subheading 3.4.**). Tryptic peptides rarely contain internal arginines, which generate complicated fragmentation patterns. The in-gel digestion procedure described in Chapter 52 is compatible with nanoelectrospray mass spectrometry when a sample desalting/concentration step is employed (**Subheading 3.5.**).

2. Materials

2.1. Equipment

1. Vacuum centrifuge.
2. Borosilicate glass capillaries (GC 120 F–10, Clark Electromedical Instruments, Pangbourne, UK).
3. Microcapillary puller (Model P-97, Sutter Instruments, Novato, California).
4. Polaron SC 7610 sputter coater (Fisons Instruments, East Sussex, UK).
5. *x-y-z* Manipulator.
6. API III triple quadrupole instrument (Perkin-Elmer/Sciex, Toronto, Canada).
7. Mini-centrifuge: PicoFuge (Stratagene, Palo Alto, CA).

2.2. Reagents

1. Poros R2 material (PerSeptive Biosystems, Framingham, MA).
2. 5% Formic acid.
3. 50% Methanol.

3. Methods

3.1. Setting Up a Nanoelectrospray Ion Source

The nanoelectrospray source consists of a metal-coated glass capillary whose tip is pulled into a needle shape with an outer diameter of about 2 μm and orifice diameter of 1 μm (**Fig. 1**).

1. The nanoelectrospray needles are made from borosilicate glass capillaries. The desired needle shape is obtained by using a two-stage pulling cycle on a microcapillary puller. Parameter set [heat, pull, velocity, time]: 1. [520, 100, 10, 200]; 2. [490, 160, 12, 165]) (*see* **Note 1**).
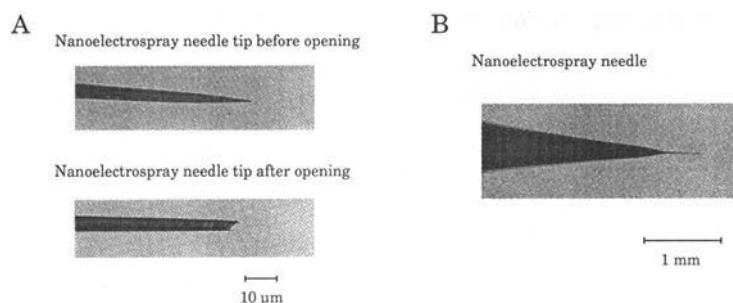
Fig. 1. Nanoelectrospray needles. **(A)** Pulled glass capillary needle tip before and after opening. The needle tip should be 200–500 μm long with an opening of only 1–2 μm. **(B)** A two-stage heat/pull cycle on a capillary puller produces two identical glass capillary needles with a thin, tapered needle tip.
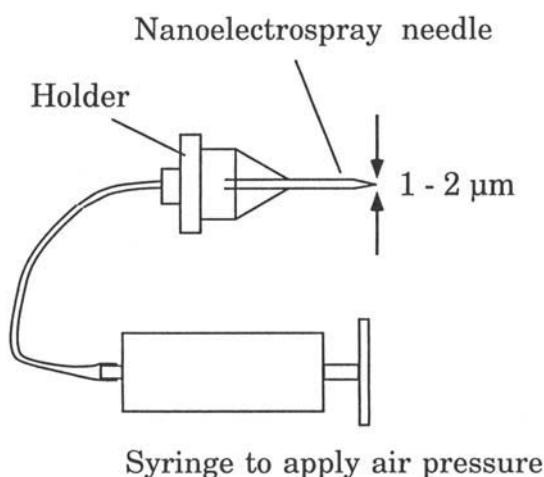
Fig. 2. Nanoelectrospray ion source. The nanoelectrospray needle is mounted in a metal holder, which is connected to the ion source power supply and to a syringe that provides the air back-pressure. The needle assembly is mounted onto an *x-y-z* manipulator, and positioned on axis and 1.5–2 mm from the orifice of the mass spectrometer.

2. Metal coating of the glass capillaries is achieved by metal (gold) vapor deposition in a sputter chamber (*see* **Note 2**).
3. The glass capillary is mounted in a gas-tight holder, which can be pressurized by air up to about 1 bar (**Fig. 2**).
4. The needle assembly is connected to the ion source power supply. Holder and needle are electrically connected by a small droplet of conductive carbon cement (Neubauer Chemikalien, Münster, Germany). The metal coating of the glass capillary needle ensures that the electrical potential is transferred to the liquid sample at the needle tip.

3.2. A Step-by-Step Guide for Installing the Nanoelectrospray Source

1. Mount the pulled and metal-coated nanoelectrospray needle in the holder. Connect the holder to the ion source power supply.
2. Make electrical contact between the needle and the holder by applying a droplet of graphite paste. (Alternatively, the power supply can be connected directly to the needle with a clamp.)
3. Mount the ion source on a *x-y-z* manipulator in front of the mass spectrometer.
4. Dissolve proteins and peptides in 5% formic acid in 20–50% methanol, and inject 0.5–2 μL into the nanoelectrospray needle using a micropipet with a gel loader tip (*see* **Note 3**). Liquid injected into the needle is drawn to the tip by capillary force.
5. Connect the needle holder to the 20-mL syringe, which provides the air pressure.
6. Position the nanoelectrospray needle on axis and 1.5–2 mm from the orifice of the mass spectrometer. Monitor the position of the needle tip with a microscope or a video camera.
7. Gently pressurize the needle by air using the 20-mL syringe.
8. If no liquid appear at the needle tip, then briefly and gently touch it against the interface plate of the mass spectrometer under microscopic control. The needle should not be visibly shortened, but a small sample droplet may appear after the contact indicating the opening of the needle tip (*see* **Fig. 1** and **Note 4**).
9. Reposition the needle in front of the orifice of the mass spectrometer.
10. Apply the voltage to needle/holder and mass spectrometer interface, and start scanning the mass spectrometer (*see* **Note 5**). If there are ions in the spectrum, reduce the air pressure in the needle to the lowest value that still keeps the flow stable. If no ions appear, then refer to **Note 6** for troubleshooting tips.

3.3. Sample Preparation for Nanoelectrospray Mass Spectrometry

The nanoelectrospray ion source unfolds its full potential when the available sample is concentrated to 1 μL or less. Centrifugal microcolumns can be used to desalt and concentrate protein and peptide samples to μL volumes *(6,7)*. A pulled glass capillary is packed with a few hundred nanoliters of Poros resin. Working in the perfusion mode, Poros material generates only a small flow resistance when packed into a capillary. Peptide solutions are normally desalted/concentrated on Poros R2 resin, protein solutions on Poros R1 resin, and hydrophilic samples and small peptides on OligoR3 material. The pulled glass capillaries used for the columns are the same as used for nanoelectrospray emitters, but they are not sputter-coated with metal.

1. For peptide analysis, Poros R2 material is used as chromatographic resin. Remove the smallest particles by sedimenting the resin three to five times in methanol. In a 1.5-mL microcentrifuge tube, make a slurry of 10–20 μL resin in 1.2 mL methanol.
2. Mount a pulled glass capillary into a custom-made capillary holder (**Fig. 3**) or into a pierced lid of a 1.5-mL microcentrifuge tube. Use a micropipet with a gel loader tip to transfer 5 μL of resin slurry into the capillary.

Capillary, loaded with
chromatographic resin

— Window

Nano-Electrospray
Capillary

1 cm

1 mm
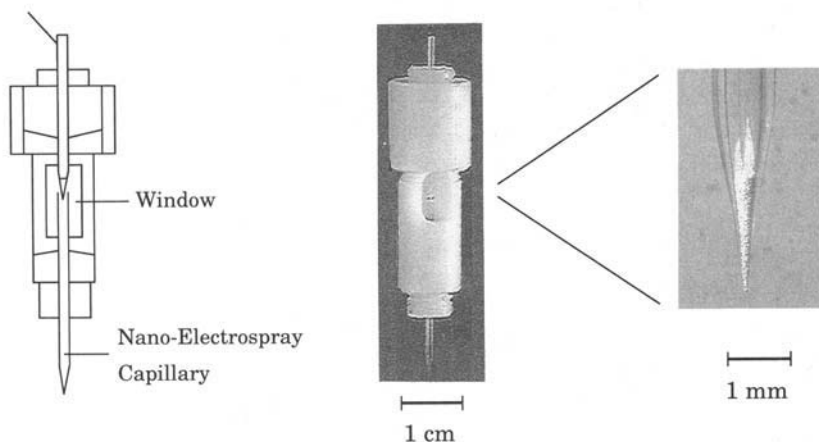
Fig. 3. Centrifugal capillary column and transfer assembly. The capillary column used for desalting/concentration of sample is made from a pulled glass capillary and filled with a small volume of Poros resin. The sample is rinsed and then eluted from the column capillary directly into the nanoelectrospray needle by gentle centrifugal force.

3. Load the chromatographic resin into the tip of the glass capillary by centrifugal force using a manually operated tabletop minicentrifuge at low speed (500–2000 rpm). The chromatographic material is visible against a dark background.

4. When sufficient chromatographic resin has been loaded into the capillary (1–2 mm resin height), the glass tip is widened/broken by gently touching it against the tabletop. The opening should allow liquid but not column resin to exit the capillary during centrifugation. Do not centrifuge the resin too fast. Otherwise it is compressed and may block the flow of liquid. The centrifugal capillary column is only used once to avoid sample-to-sample contamination.

5. Rinse the capillary column by injecting 5 μL 50% MeOH followed by gentle centrifugation.

6. Equilibrate the column resin by injecting 5 μL 5% formic acid, into the capillary followed by centrifugation until all liquid has passed through the column.

7. Dissolve the sample in 10–20 μL 5% formic acid, and inject it onto the capillary column in aliquots of 5 μL followed by centrifugation.

8. Wash the column resin twice by centrifugation with 5 μL 5% formic acid solution. This desalting step is very efficient, since the column is washed with 50–100 times its resin volume. Before beginning the elution step, the washing solution must be completely removed by gentle centrifugation.

9. Mount the capillary column in-line with a premade nanoelectrospray needle in a custom-made capillary holder that fit into a microcentrifuge (**Fig. 3**).

10. Elute the peptide mixture into the nanoelectrospray capillary by centrifuging twice with 0.5 μL 60% methanol/5% formic acid. Elute proteins with 60–70% acetonitrile/5% formic acid. This procedure allows handling of elution volumes between 10 and 0.2 μL. Elution, however, should be performed twice, because the first elution does not completely deplete the column. Keep in mind that signal intensity in an electrospray spectrum is concentration dependent so keep the elution volume as small as possible.

11. Mount the loaded nanoelectrospray needle onto the ion source and begin the experiment (*see* **Note 9**).

3.4. Nanoelectrospray Tandem Mass Spectrometry of Unseparated Peptide Mixtures

Peptide sequencing with tandem mass spectrometry *(15,16)* consists of three steps:

1. Measuring the *m/z* values of peptides in a sample.
2. Acquiring the tandem mass spectra after collision-induced dissociation (i.e., fragmentation) of selected peptides.
3. Interpreting the tandem mass spectrometry data.

With the nanoelectrospray source, the first two steps are performed in one experiment with the unseparated peptide mixture.

The *m/z* values of analyte peptides are detected by comparing a Q_1 mass spectrum to a representative spectrum of the autolytic peptides of the enzyme used (i.e., trypsin) or a representative control from a particular experiment. It is often advantageous to process an empty gel piece excised near the protein band of interest as control (*see* Chapter 52).

At subpicomole protein amounts on the gel, we routinely employed the parent ion scan technique to detect peptide ion signals that were below the chemical noise in the normal Q_1 spectrum (**Fig. 4**) *(17)*. Parent ion (or precursor ion) scans of the abundant *m/z* 86 immonium ion of isoleucine/leucine detect peptides that contain these common amino acids. For the parent ion scan, the mass spectrometer parameters are adjusted to obtain optimum detection efficiency at reduced mass resolution. The parent ion scan technique can also be used to detect selectively phosphopeptides by monitoring *m/z* 79 in the negative ion mode *(17–19)*, to detect selectively glycopeptides by monitoring *m/z* 162 or 204 in the positive ion mode *(17,20)*, and to detect intact proteins or oligonucleotides in contaminated samples *(21)*.

Once a set of peptide *m/z* values has been determined by either Q_1 scans or by parent ion scans, high-resolution scans can be performed for selected peptide ion signals in the "multiple ion monitoring" mode to determine the exact peptide mass and the peptide charge state based on the isotope spacing. The reduction of sensitivity when measuring at high resolution is compensated by adding many scans, for example, 50 or more, to one spectrum. The latter fea-
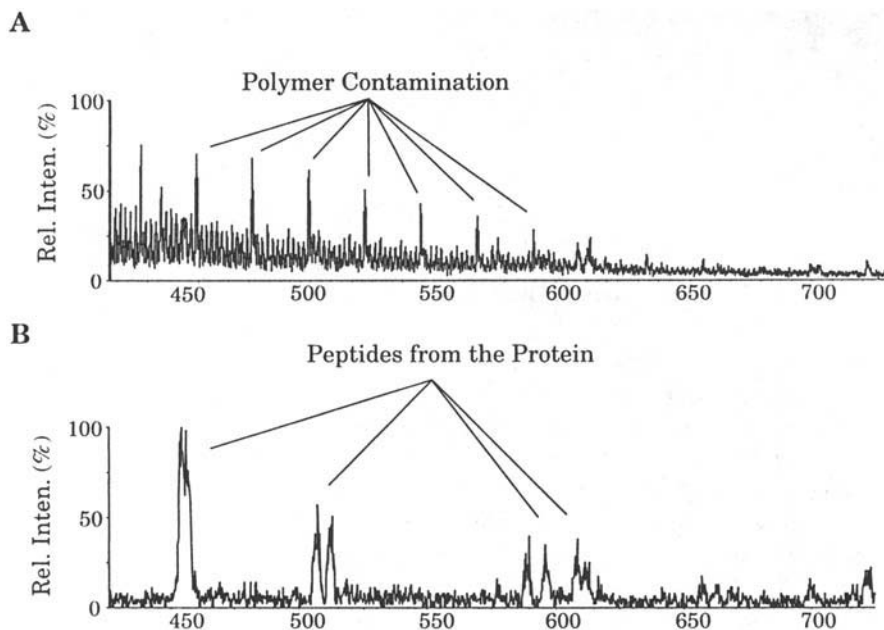
Fig. 4. Parent ion scan for the leucine/isoleucine immonium ion (*m/z* 86) detects peptide ions below the chemical noise level. **(A)** The nanoelectrospray mass spectrum (Q_1 scan) of a tryptic peptide mixture displays only polymer ion signals and chemical background, which suppress peptide ions. **(B)** The parent ion (*m/z* 86) analysis of the same sample reveals several peptide ion signals, which subsequently can be selected for sequencing by tandem mass spectrometry.

ture further demonstrates the utility of long measurement times that the nanoelectrospray source provides. It is advantageous to select doubly charged tryptic peptide ions for tandem mass spectrometry experiments, because they generate relatively simple fragment ion spectra. Triply charged tryptic peptides can also be fragmented and often allow determination of long stretches of amino acids sequence, i.e., 15–25 consecutive residues, via doubly charged fragment ion series.

3.5. Fragmenting Peptides by Collision-Induced Dissociation

Once a set of peptide *m/z* values has been accurately determined, each peptide is fragmented in turn. For peptide sequencing by tandem mass spectrometry using a triple quadrupole instrument, two main instrumental parameters are adjusted to obtain high-quality amino acid sequence information. First, the resolution setting of the first quadrupole (Q_1) is adjusted according to the abundance of the peptide ion signal, i.e., the lower the ion intensity, the higher the
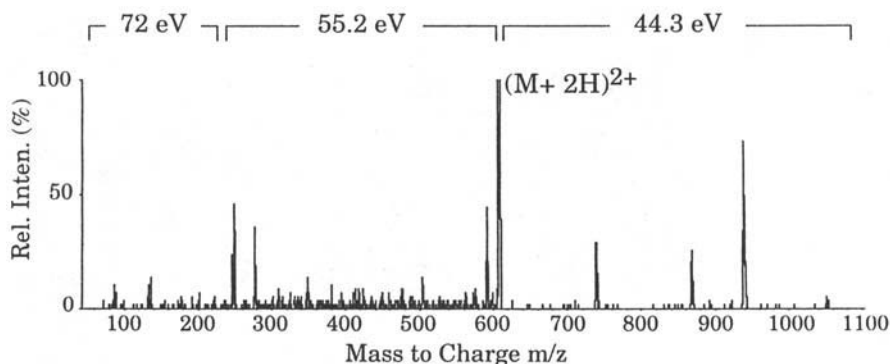
Fig. 5. Peptide tandem mass spectrum acquired in separate segments. The *m/z* range above the precursor ion, $[M + 2H]^{2+}$, is acquired at low collision energy and at relatively low mass resolution to generate and detect large peptide fragment ions efficiently. The *m/z* range below the precursor ion is acquired at higher resolution and with stepped collision energies, i.e., intermediate collision energy to generate low *m/z* sequence ions and high collision energy to generate immonium ions and the a_2 and b_2 fragments.

resolution setting in order to reduce the chemical background noise in the lower half of the tandem mass spectrum for a better signal-to-noise ratio. Second, the collision energy can be adjusted according to the peptide mass and varied depending on the mass range scanned (**Fig. 5**). The collision gas pressure is kept constant throughout the MS/MS experiment.

It may be advantageous to acquire a tandem mass spectrum in two or three segments. The high *m/z* segment is acquired with a wide parent ion selection window (low resolution) and a low collision energy to generate and detect relatively large peptide ion fragments. The low *m/z* region is acquired at higher resolution and at higher collision energies to generate and detect low *m/z* fragments and immonium ions. The nanoelectrospray allows this and other types of optimization owing to the stability and long duration of the spray.

When investigating a peptide mixture by tandem mass spectrometry, as many peptides as possible should be fragmented. This motivated the development of semiautomatic software routines to assist in data acquisition. The list of peptide *m/z* values is stored by customized software that calculates the optimum hardware settings for subsequent sequencing of each individual peptide. However, for *de novo* sequencing of long stretches of amino acid sequences, it is not yet advisable to use automated software routines for data acquisition. Careful adjustments of collision energy and mass resolution is required to obtain high-quality data for unambiguous sequence assignments. Only when very high data quality is obtained, e.g., when employing a quadrupole time-of-flight tandem mass spectrometer in combination with ^{18}O-labeling *(22)*, are software routines reliable.
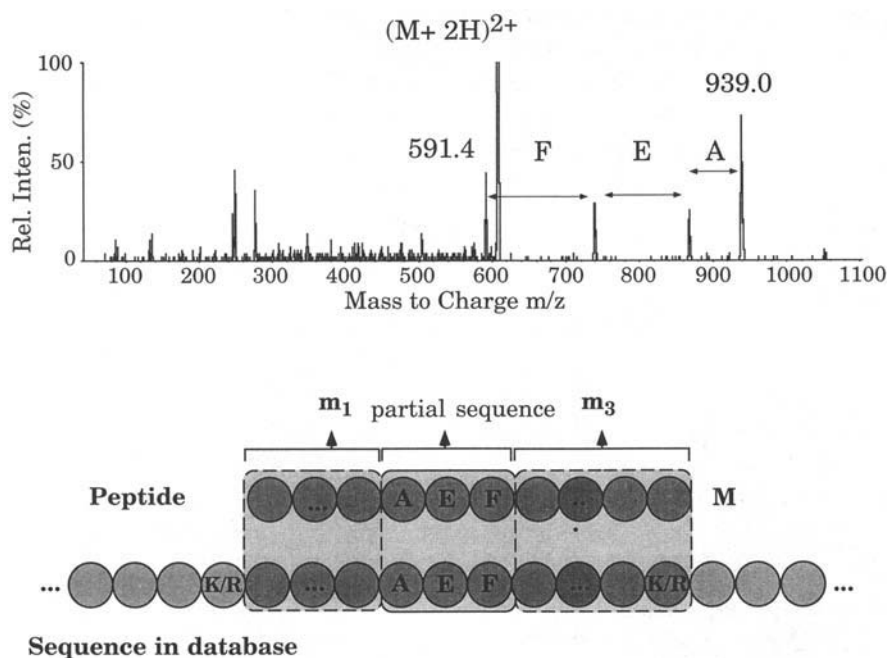
Fig. 6. Peptide sequence tag generated from a tandem mass spectrum. A short search string, (591.4)FEA(939.0), is readily identified in the tandem mass spectrum of this tryptic peptide with mass M. It is subsequently converted to a peptide sequence tag by the *PeptideSearch* software and used to query a database. The modular composition of a peptide sequence tag permits error-tolerant searches where one or two of the modules are allowed to contain an error.

3.6. Generation of Peptide Sequence Tags from Tandem Mass Spectra of Peptides

Complete interpretation of tandem mass spectra of peptides can be complicated and requires some experience. However, it is often relatively straightforward to generate short sequences of two to five amino acid residues from a tandem mass spectrum. This information is valuable for sequence database searches as follows. A "peptide sequence tag" is assembled from the peptide mass, a short internal sequence of amino acid residues, and the distance in mass to the N- and C-terminus of the peptide *(23)* (**Fig. 6**). The search specificity of this construct is very high, because the amino acid sequence is "locked" in place by the masses of the "unknown" parts of the peptide. The modular composition of a peptide sequence tag makes it tolerant to errors in any one of the modules. Since only a fraction of the information content of the tandem mass spectrum is used to generate a peptide

sequence tag used to query the sequence database, the remaining information confirm a retrieved peptide sequence: Every significant fragment ion signal should correlate to the peptide sequence. A peptide sequence tag consisting of three residues typically retrieve only one protein from a database containing more than 200,000 sequences. If longer stretches of sequence can be read out of a tandem mass spectrum, i.e., six or more residues, it is advantageous to search by amino acid sequence instead of by peptide sequence tags. Searching by amino acid sequence is more flexible and allows sequence homology searches.

As mentioned above, tryptic peptides have the desirable feature that they contain an N-terminal amino group and a C-terminal Lys or Arg residue, localizing protons at both the N-terminus and the C-terminus of the peptide. Tandem mass spectra of tryptic peptides very often contain a continuous y-ion series, which can be readily assigned in the *m/z* range above the doubly charged parent ion signal. Our spectrum interpretation strategy builds on this characteristic. It is guided by the demand to identify a protein in sequence databases or to sequence peptides reliably for cloning of the cognate protein.

The following list summarizes a few basic empirical rules that we use in interpreting tandem mass spectra of tryptic peptides. Since peptides differ in their fragmentation behavior in a sequence-dependent manner, it is possible to find exceptions to these rules.

1. The goal of the interpretation is to find a series of peaks that belong to one ion series—for tryptic peptides mostly y-ions (C-terminal fragments).
2. Initial peak selection: The high *m/z* region of a tandem mass spectrum is often straightforward to interpret. Choose an intense ion signal in this region as the "starting peak."
3. Assembly of a partial amino acid sequence: Try to find ion signals that are precisely one amino acid residue mass away from the starting peak (up or down in mass). We use software that marks all the possibilities in the spectrum. This provides a good overview whether there is more than one possibility for sequence assignments. If there is a repeating pattern of fragment ion peaks with satellite peaks representing an H_2O loss (–18 Dalton) or an NH_3 loss (–17 Dalton), a fragment ion series has been identified (for tryptic peptides a y-ion series is more likely).
4. By repeating **step 3**, a peptide sequence tag consisting of two to four amino acids is assembled, which is subsequently used to identify a protein in the sequence database. *PeptideSearch* software automatically assembles a sequence tag from a series of fragment ion masses *(23,24)*. As default for tryptic peptides, the database is searched under the assumption that a y-ion series was determined. However, even for tryptic peptides, the tandem mass spectrum can be dominated by b-ions if a peptide contained an internal basic residue or when the C-terminal peptide of the protein had been sequenced.

3.7. Confirming Protein Identifications Made by Peptide Sequence Tags

If a protein sequence is retrieved by a database search with a peptide sequence tag, then the amino acid sequence of the retrieved peptide should fit the tandem mass spectrum in order to be called a positive match. Two or more peptides from a sample should independently identify the same protein in a database. To verify a match, the peptide fragment masses must be correct within the expected error of the mass measurement. For tryptic peptides, the y-ion series should be nearly complete, except when a peptide contains an internal proline residue *(see below)*. The N-terminal b_2 and a_2 fragment ions, generated at relatively high collision energy, should be present in the low *m/z* region. Odd fragmentation patterns should reflect the amino acid sequence as discussed in the following paragraphs.

Peptides that contain internal basic residues (lysine or arginine) do not fragment in the vicinity of these residues, because a charge is localized at the side chain of the basic residue and therefore not available for amide backbone cleavage. If the triply charged precursor ion was fragmented, then doubly charged y-ions are present in the spectrum.

Internal proline residues deserves special attention. Cleavage of the C-terminal bond of a proline is observed to a low degree. The N-terminal bond of a proline is labile, giving rise to an intense y-ion fragment. Internal fragmentation of peptides containing a proline often confirms a sequence: The y-ion generated by fragmentation at the N-terminal side of Pro will dissociate a second time to produce a y-ion series, which is superimposed on the original y-ion series. However, the internal immonium ions (b-type ions) generated from this Pro-containing peptide fragment serve to confirm the C-terminal part of a peptide sequence up to and including the internal proline residue.

Isoleucine and leucine cannot be differentiated by amide bond cleavage alone, because they have the same elemental composition and therefore identical molecular weight. Pairs of amino acid with identical nominal mass, Lys/Gln (128 Dalton) and oxidized Met/Phe (147 Dalton) can often be distinguished. Lys and Gln differ in their basicity, and trypsin cleaves C-terminal to Lys and not at Gln, so internal Lys is rarely found in tryptic peptides. However, if the latter is the case, then the tryptic peptide usually acquires an additional proton for a total of three charges, and the tandem mass spectrum will often contain a doubly charged y-ion series. If an internal Lys residue is suspected, then the peptide mixture can be inspected for the presence of the limit peptide produced by tryptic cleavage at this Lys residue. Oxidized methionine (147.02 Dalton) and phenylalanine (147.07 Dalton) residues are differentiated relatively easily as follows. Tandem mass spectra of peptides that contain an oxidized methionine residue (i.e., methionine sulfoxide) display satellite peaks, which appear 64 Dalton

below each methionine sulfoxide-containing y-ion fragment owing to loss of CH_3SOH from methionine sulfoxide *(25,26)*. The oxidation reaction is often not complete, so inspection of the peptide mass spectrum (Q_1 spectrum) may reveal a peptide ion signal 16 Dalton below the one containing oxidized methionine.

If no proteins are retrieved with a simple database search, then search under the assumption that some of the amino acids of the peptide are modified, for example, oxidized methionine or *S*-acrylamidocysteine. An error-tolerant search can be launched in which only partial correspondence between the peptide sequence tag and a database entry is required *(23)*. Additional information about the protein can be used to select possible candidates if more than one protein sequence is retrieved (such as protein size, organism, or function).

If a protein cannot be identified by any of its peptide sequence tags, we conclude that it is unknown. For proteins from human, mouse, or other model organisms, the peptide sequence tags are then screened against a database of expressed sequence tags (ESTs) *(27)*. ESTs are short stretches of cDNA, i.e., single-stranded DNA generated from expressed mRNA by reverse transcriptase, and thus represents the set of expressed genes in a given cell line. If a database search retrieves a cDNA sequence, then library screening and cloning are relatively straightforward. If the EST database search produces no hit, then *de novo* peptide sequencing has to be pursued *(10)*.

3.8. De Novo Peptide Sequencing
by Nanoelectrospray Tandem Mass Spectrometry

To sequence unambiguously an unknown protein for homology searching and cloning, two data sets are usually required *(10,15)*. The tryptic peptide mixture is split in two portions. The first portion of the mixture is analyzed by nanoelectrospray tandem mass spectrometry, and long peptide sequences are generated through complete interpretation of tandem mass spectra using the guidelines described above. The other portion of the peptide mixture is *O*-methyl-esterified *(15,28)* and then analyzed. Every free carboxyl-group, including the C-terminus of peptides, is esterified and therefore increases in mass by 14 Dalton. The number of methyl-esters in a peptide can be determined by the mass shift of peptides, which is predictable from the previously interpreted tandem mass spectra of the native peptides. Because all y-ion fragments produced from an esterified peptide contain the C-terminus, they are all shifted up in mass. Comparison of a set of tandem mass spectra obtained from a peptide and the corresponding esterified peptide serve to confirm the amino acid sequence, because the y-ion series can unambiguously be assigned (**Fig. 7**). Additionally, internal acidic residues, Asp and Glu, are methylated as well and can easily be differentiated from their corresponding amide residues, Asn and Gln, which otherwise differ in mass by only 1 Dalton.

Unmodified Peptide
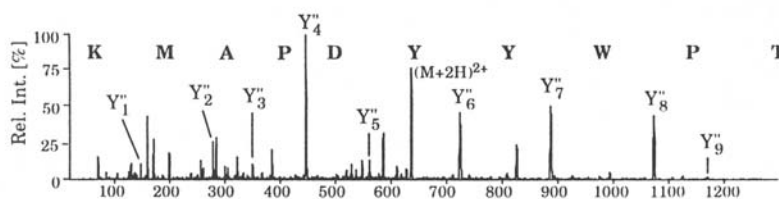
Esterified Peptide
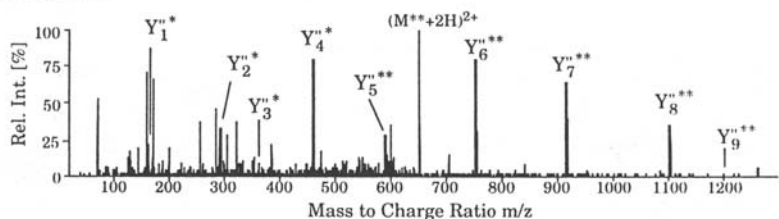
Fig. 7. Tandem mass spectra of **(A)** native and **(B)** *O*-methylesterified peptide. Methylation of peptide carboxyl groups results in a mass increase of 14 Dalton of all peptide fragment ions that contain acidic residues, including the C-terminal y-ions. This approach identifies y-ion series and resolves Asp/Asn and Glu/Gln ambiguities. The number of acidic residues in peptide ion fragments is indicated with asterisks.

An alternative method to recognize y-ion series employs ^{18}O-labeled water (*see*, for example, ***29,30***). By performing the trypsin digestion in a 1:1 mixture of normal water and ^{18}O-labeled water, all the tryptic peptides will incorporate ^{18}O at the C-terminus with a yield of approx 50%. Each peptide ion appears in a mass spectrum as a doublet separated by 2 Dalton. Selecting both isotope species together for fragmentation (low-resolution setting in Q_1 precursor ion selection) produces tandem mass spectra that display y-ions as a series of split peaks, i.e., separated by 2 Dalton, whereas b-ions are single peaks. This aids in the interpretation of a peptide tandem mass spectrum, because y-ion series are unambiguously assigned. This approach is very attractive when using a hybrid quadrupole-TOF tandem mass spectrometer, which produces highly resolved fragment ion signals ***(22)***. Note that the ^{18}O-labeled water has to be very pure to avoid chemical background noise. Redistillation of commercially available ^{18}O-labeled water is recommended.

3.9. O-Methylesterification of Peptides

This approach works well for methyl esters only. Peptide esterification by ethyl alcohol is incomplete.

3.9.1. Preparation of Reagent

1. Cool 1 mL of distilled (i.e., dry) methanol in a 1.5-mL microcentrifuge tube in the freezer (–20 or –80°C) for 10–15 min.
2. Dropwise add 100 μL of acetyl chloride. Beware that the reaction mixture may react violently and spray liquid around (or on) you.
3. Allow the reagent cocktail to warm up to room temperature, and use it 10 min later.

3.9.2. Derivatization

1. Dry the peptide (or peptide mixture) in a vacuum centrifuge. Add 2–5 μL of the reagent. If the sample contains salts, then add enough reagent to cover the solid residue at the bottom of the tube.
2. Incubate at room temperature for 30 min.
3. Dry the sample in a vacuum centrifuge.
4. Esterified peptides can be redissolved in 5% formic acid. They are relatively stable under acidic conditions, but can undergo rearrangement at mild alkaline pH.

4. Notes

1. The first heating/pulling stage reduces the diameter of the capillary to about 0.5 mm, but the second stage pulls the glass capillary apart, producing two nanoelectrospray needles. These needles should have an opening of 1–2 μm. However, after pulling, the opening diameter can be <100 nm and has to be widened (**Subheading 3.2.**). To reduce the flow resistance for a stable flow rate in the 10–25 nL/min range, the narrow part of the tip should not be longer than 500 μm (**Fig. 1**). Needles with very short constrictions (50–100 μm) can be operated easily, but with a higher risk of loosing sample owing to a higher flow rate. We prefer such short needles for rapid mass measurements when abundant sample is available, e.g., recombinant proteins, synthetic peptides, or oligonucleotides. Longer tips (200–500 μm) are used for tandem mass spectrometry experiments when the longest possible operation time is desirable and when the sample load volume will not exceed 1 μL. A major advantage of these types of nanoelectrospray needles is that they do not easily block owing to the relatively short length of the needle tip. Nanoelectrospray needles and ion sources are commercially available from Protana A/S (Odense, Denmark).
2. The needles are only used once, so it is not a problem that the coating is not tightly fixed to the glass and can be rubbed off. Methods to produce a more stable metal coating include pretreatment with (3-mercaptopropyl)trimethoxysilane *(31)* or protecting the metal layer by a second layer of SiO_x *(32)*. A stable gold coating is necessary when a glass needle is used for several samples over a prolonged time. The needle tip is fragile, so take care not to break it when loading sample and mounting the needle in the holder.
3. The electrospray generated with the nanoelectrospray ion source is very stable. This allows purely aqueous solutions to be sprayed even in negative ion mode without nebulizer assistance. The source can be operated with solutions contain-

ing up to 1 *M* NaCl *(6)*, although this is not recommended. The high stability allows optimization of experimental conditions based on analyte characteristics rather than electrospray requirements, i.e., when choosing buffer composition. For example, preservation of noncovalent complexes often does not allow addition of organic solvent to the sample to facilitate spraying. Because the ion source exclusively produces very small droplets, relatively soft desolvation conditions in the interface region of the mass spectrometer can be chosen.

4. Needle and plate should be at the same electric potential. When the needle tip is opened, a tiny droplet may appear on the metal plate—it spreads out as a faint shadow in a few seconds.

5. The main objective when operating the nanoelectrospray source is to achieve a low and stable flow rate despite sample-to-sample variations. This is achieved by applying air pressure to the needle, which helps to adjust the flow rate and thereby compensates for differences in needle orifice size and sample viscosity. The flow rate of a nanoelectrospray source is about 25 nL/min. At this flow rate, analyte concentrations of 1 pmol/μL results in one analyte molecule per droplet on the average *(5)*. Furthermore, 1 μL of sample is consumed in 40 min, extending the time available for optimization of experiments. The overall sensitivity is limited by the signal-to-noise level, and it is therefore a function of the ionization efficiency, desolvation efficiency, ion transmission, the level of chemical background ions, and detector characteristics. Several parameters were changed in the inlet region of the mass spectrometer in order to operate the nanoelectrospray ion source. These changes are described below:

   a. The nanoelectrospray source is mounted directly in front of the orifice of the mass spectrometer, on axis with the mass analyzer, and at a distance of 1.5–2 mm from the orifice. Electrospray at a low flow rate generates very small droplets with a diameter of 200 nm or less. Desolvation is therefore achieved in a very short time and distance.

   b. To initiate the electrospray, a minimal electrical field strength at the surface of the liquid has to be reached *(33)*. Conventional electrospray ion sources are operated with a 3- to 5-kV potential difference between the needle and counterelectrode (i.e., the orifice plate of the mass spectrometer). The very small tip diameter of the nanoelectrospray needle allows a spray cone to be established at a much lower electrical potential, typically 400–700 V.

   c. Because the charged droplets generated by the nanoelectrospray ion source are very small, softer desolvation conditions in the interface (skimmer) region of the mass spectrometer are used. The electrical gradient and the countercurrent gas flow can be reduced. This appears to lead to the generation of colder molecular ions as compared to conventional electrospray sources facilitating, for example, studies of noncovalent molecular interactions.

   The API III triple quadrupole instrument (Perkin-Elmer/Sciex) has a channeltron detector, which is operated in single-ion counting mode. At a concentration of usually 10 fmol/μL, the limit for peptide detection in Q_1 scan mode (*m/z* 400–1500) with a signal-to-noise ratio larger than 1 is reached. Using parent or precursor ion

scan techniques, the sensitivity can be extended to 1 fmol/µL. Detection of proteins requires higher analyte concentration, because the ion current is spread over multiple charge states and the molecules are more difficult to desolvate.

6. The nanoelectrospray ion source is a very robust device. However, problems may occur if the sample contains high concentrations of buffers, polymers, or salts, or if the shape of the needle tip is not within the dimensions described above. In this section, we provide a few troubleshooting tips. If there are no ions or no noise in the spectrum, then the needle is not spraying. Repeat **Subheading 3.3.**, **step 8** to open the needle tip. If the spray becomes instable, then jitter and spikes will appear in the spectrum or the spectrum will contain an unusually high level of chemical background. Increasing the air pressure of the needle helps stabilize the flow. If this simple measure is insufficient, then the spraying voltage may be too low (increase it by 100–150 V) or the opening of the needle tip is still too small (repeat **Subheading 3.3.**, **step 8**). Be aware that the nanoelectrospray needle has a very small diameter. Small changes in the applied potential (increases of 100 V) change the field density at the tip considerably. Electrical discharge can be initiated and ionize atmospheric gas, which generates a mass spectrum. These ions are usually small (<400 Dalton), and therefore, chemical background ions in the higher mass region are missing. Atmospheric gas ionization can be visible as a blue corona around the needle tip (when light sources are switched off) and may lead to oxidations of methionine-containing peptides *(34)*. If opening of a needle is not successful by the means described above, then apply voltage to the needle, pressurize it, and briefly touch it against the mass spectrometer interface plate (the potential difference between needle and interface plate should be about 500 V). The combined mechanical and electrical stress opens almost every needle. It is not advisable to open needles routinely by this approach, because it tends to damage the metal coating at the tip. High electrical current drawn from very thin tips can heat the glass to a degree that the glass melts. The damaged piece of the tip can often be broken off, and analysis can proceed with a larger opening. Two effects can prevent or stop spraying from an opened needle: the high surface tension of the liquid or precipitation of salts, polymers, or other nonvolatile sample constituents when their concentration is high. In the former case, the needle can easily be reopened by slightly touching it to the interface plate, thereby destroying the surface tension by physical contact. In the latter case, the harsher procedure employing mechanical and electrical stress for needle opening (described above) is perhaps required.

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Index

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2-D Proteome Analysis Protocols

Edited by

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In *2-D Gel Proteome Analysis Protocols*, Andrew Link and his expert collaborators take today's researchers step-by-step through the complete process of doing proteomics. With easy-to-follow instructions, complete with many helpful hints and explanations, leading investigators and pioneers in the field show how to make protein extracts, reproducibly run them on 2-D gels, detect them, analyze the data, and precisely identify each protein. The book covers the latest methods of using carrier ampholytes in the 1st dimension, casting and running immobilized pH gradient 2-D gels, MALDI-TOF-based peptide mapping, automated tandem mass spectrometry, and nanoelectrospray ionization technology. For the 2nd dimension, there are methods for running flatbed or vertical gels and for protein detection using autoradiography, and Coomassie, silver, and reversible metal-chelate stains. The book is a perfect complement to the genome sequencing project for answering biological questions.

2-D Gel Proteome Analysis Protocols is the most complete guide for using proteomics to answer biological questions. Whether it is a question of global protein analysis or evaluating a cell's response to internal or external stimuli, the advanced methods described here will enable today's researchers better to understand how cells work and open new possibilities for drug discovery.

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- **Proven protocols for characterizing and identifying the global expression of proteins**
- **Vital tools for drug discovery and drug targeting**
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- **Protocols for constructing 2-D databases for the WWW and comparing 2-D gels over the Internet**
- **Mass spectrometry methods for rapid, high-sensitivity protein identification**
- **Detailed techniques for acquisition and computer analysis 2-D gel images**

CONTENTS

2-D Protein Gel Electrophoresis: *An Overview*. Solubilization of Proteins in 2-D Electrophoresis: *An Outline*. Preparation of *Escherichia coli* Samples for 2-D Gel Analysis. Preparing 2-D Protein Extracts from Yeast. 2-D Protein Extracts from *Drosophila melanogaster*. Preparing 2-D Protein Extracts from *Caenorhabditis elegans*. Eukaryotic Cell Labeling and Preparation for 2-D. Differential Detergent Fractionation of Eukaryotic Cells: *Analysis by Two-Dimensional Gel Electrophoresis*. Fractionated Extraction of Total Tissue Proteins from Mouse and Human for 2-D Electrophoresis. Preparation and Solubilization of Body Fluids for 2-D. 2-D Electrophoresis of Plant Proteins. Quantifying Protein in 2-D PAGE Solubilization Buffers. Measuring the Radioactivity of 2-D Protein Extracts. Advantages of Carrier Ampholyte IEF. 2-D Electrophoresis Using Carrier Ampholytes in the First Dimension (IEF). Nonequilibrium pH Gel Electrophoresis (NEPHGE). High-Resolution, 2-D Protein Electrophoresis Using Nondedicated Equipment. Large-Gel 2-D Electrophoresis. Advantages of Immobilized pH Gradients. Casting Immobilized pH Gradients (IPGs). Analytical IPG-Dalt. IPG-Dalt of Very Alkaline Proteins. Running Preparative Carrier Ampholyte and Immobilized pH Gradient IEF Gels for 2-D. In-Gel Sample Rehydration of Immobilized pH Gradient. High-Resolution, IPG-Based, Mini Two-Dimensional Gel Electrophoresis,. Horizontal SDS-PAGE for IPG-Dalt. Casting and Running Vertical Slab-Gel Electrophoresis for 2D-PAGE. Nonreducing 2-D Polyacrylamide Gel Electrophoresis. 2-D Diagonal Gel Electrophoresis. 2-D Phosphopeptide Mapping. Internal Standards for 2-D. Autoradiography of 2-D Gels. Double-Label Analysis. Silver Staining of 2-D Electrophoresis Gels. Staining of Preparative 2-D Gels: *Coomassie Blue and Imidazole-Zinc Negative Staining*. Electroblotting of Proteins from 2-D Polyacrylamide Gels. Detection of Total Proteins on Western Blots of 2-D Polyacrylamide Gels. Protein Detection Using Reversible Metal Chelate Stains. Gylcoprotein Detection of 2-D Separated Proteins. Image Acquisition in 2-D Electrophoresis. Computer Analysis of 2-D Images. 2-D Databases on the World Wide Web. Comparing 2-D Electrophoresis Gels Across Internet Databases. Constructing a 2-D Database for the World Wide Web. Absolute Quantitation of 2-D Protein Spots. Generating a Bacterial Genome Inventory: *Identifying 2-D Spots by Comigrating Products of the Genome on 2-D Gels*. Immunoaffinity Identification of 2-DE Separated Proteins. 2-DE Spot Amino Acid Analysis with 9-Fluroenylmethyl Chloroformate. N-Terminal Amino Acid Sequencing of 2-DE Spots. Characterizing Proteins from 2-DE Gels by Internal Sequence Analysis of Peptide Fragments: *Strategies for Microsample Handling*. Obtaining Molecular Weights of Proteins and Their Cleavage Products by Directly Combining Gel Electrophoresis with Mass Spectrometry. Identification of Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using Peptide and Fragment Ion Masses. Sample Preparation Methods for Mass Spectrometric Peptide Mapping Directly from 2-DE Gels. Protein Identification and Analysis Tools in the ExPASy Server. Automated Protein Identification Using Microcolumn Liquid Chromatography-Tandem Mass Spectrometry. Peptide Sequencing of 2-DE Gel-Isolated Proteins by Nanoelectrospray Tandem Mass Spectrometry. Index.

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