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Daniel R. Perez *Editor*

Reverse Genetics of RNA Viruses

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Edited by

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Preface

The International Committee on Taxonomy of Viruses (ICTV) classifies RNA viruses as those that belong to Group III, Group IV, or Group V of the Baltimore classification system and contain ribonucleic acid (RNA) as genetic material throughout their entire life cycle. Group III includes double-stranded RNA viruses (dsRNAs), whereas Groups IV and V contain single-stranded RNA viruses (ssRNAs) of positive and negative polarity, respectively. Positive sense RNA viruses (+ssRNAs) are those in which the RNA itself is translated by the host cell translation machinery and initiates an infectious cycle de novo. In contrast, negative sense RNA viruses (−ssRNAs) cannot be translated directly and require copying of the negative sense RNA into a positive sense RNA strand before the infection can proceed.

In biology, the term "forward genetics" is used to define an approach that seeks to find the genetic basis of a phenotype or trait. Forward genetics of RNA viruses implies imposing them to various stress conditions and then defining the genetic changes that occurred in the process. The term "reverse genetics" is an approach to unravel the function of a gene by establishing and analyzing the phenotypic effects of (artificially) engineered gene sequences. In case of RNA viruses, reverse genetics invariably requires the de novo reconstitution of the virus from a cDNA copy. Using molecular biology, cDNA copies of RNA viruses are cloned into a variety of vectors, most typically and in order of preference, plasmids, bacterial artificial chromosomes or bacmids, or recombinant viral vectors. The ability to further manipulate DNA elements encoding portions or entire cDNA copies of RNA viruses has revolutionized the manner in which these viruses can be studied and understood. Thanks to reverse genetics, it is possible to better define the molecular mechanisms that modulate pathogenesis, transmission, and host range of RNA viruses, to study virus evolution, receptor binding characteristics, virus entry, replication, assembly, and budding. Reverse genetics allows the development of novel vaccine strategies and to better test and/or develop alternative intervention strategies such as novel antivirals. Perhaps the initial perception is to think that reverse genetics of dsRNAs and +ssRNAs is easier than −ssRNAs; however, genome size, secondary RNA structures, genome segmentation, cryptic signal sequences, among other issues, make reverse genetics of all kinds of RNA viruses equally challenging.

This book *Reverse Genetics of RNA Viruses: Methods and Protocols* is a compilation of 16 chapters summarizing reverse genetics breakthroughs and detailed reverse genetics protocols. The book does not cover every reverse genetics protocol for every RNA virus. Instead, it does provide comprehensive protocols for those RNA viruses that were initially the most challenging to obtain and/or that were developed most recently. This book, of course, would not have been possible without the outstanding and most generous contributions of our authors who are leaders in their respective fields and that have shared their insights and step-by-step protocols to help you, our colleagues, with your own research endeavors. I hope you find this book helpful.

Athens, GA, USA ***Daniel R. Perez***

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Chapter 1

Reverse Genetics for Mammalian Orthoreovirus

Johnasha D. Stuart*, Matthew B. Phillips*, and Karl W. Boehme

Abstract

Reverse genetics allows introduction of specific alterations into a viral genome. Studies performed with mutant viruses generated using reverse genetics approaches have contributed immeasurably to our understanding of viral replication and pathogenesis, and also have led to development of novel vaccines and virus-based vectors. Here, we describe the reverse genetics system that allows for production and recovery of mammalian orthoreovirus, a double-stranded (ds) RNA virus, from plasmids that encode the viral genome.

Key words Plasmid-based reverse genetics, Reovirus, Double-stranded RNA virus, Recombinant virus, Viral reassortment, T7 RNA polymerase

1 Introduction

Viral mutants are powerful experimental tools. Analysis of mutant viruses has produced myriad breakthrough in our understanding of viral pathogenesis by illuminating how viruses replicate, alter host cell physiology, and modulate immune responses. Viral mutants can be derived using "forward genetics," where a selective pressure impairs one or more viral functions and requires the virus to adapt in order to replicate efficiently under the restrictive condition. Defining genetic changes that occur during adaptation can identify nucleotides in coding or noncoding regions of the viral genome that are associated with resistance to particular pressures. Forward genetics approaches are extremely effective for mapping the functions of viral proteins, but requires a selective pressure to restrict the virus and force genetic changes. In contrast, the ability to engineer viruses via reverse genetics enables the testing of properties for which a selective pressure is not available. Reverse genetics is the direct introduction of specific alterations,

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including point mutations, insertions, and deletions, into a viral genome. In this chapter, we provide a protocol for generating mammalian orthoreovirus (reovirus) using a plasmid-based rescue system.

Reovirus is a member of the *Reoviridae* family of viruses that infect a range of host organisms, including mammals, birds, insects, and plants [1]. The *Reoviridae* family includes rotavirus, a common diarrheal pathogen among children [2]; bluetongue virus, an economically important agricultural pathogen that causes disease in sheep and cattle [3]; and mammalian orthoreovirus, a useful model for studies of dsRNA virus replication and pathogenesis [1]. Reoviruses were originally isolated in the 1950s [4]. Most people become infected by at least one of the three circulating reovirus serotypes during childhood [5]. Although reovirus infections are typically asymptomatic and self-resolve, they are implicated in a number of cases of central nervous system disease in children [1]. The three reovirus serotypes are represented by a prototype laboratory strain: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) [1]. Here, we provide a protocol for rescue of strains T1L and T3D using plasmid-based reverse genetics.

Reoviruses are non-enveloped, icosahedral viruses that contain a segmented genome consisting of ten ds RNAs [1]. The genomic dsRNA molecules are divided into three categories based on their molecular weight [6, 7]. The reovirus genome contains three large (L), three medium (M), and four small (S) genomic segments [8]. Each gene segment encodes a single viral protein except for the S1 segment, which encodes two proteins. The 5′ end of each reovirus positive-sense RNA contains a 7-methylguanosine cap, but the 3′ termini are not polyadenylated [9]. The negative-sense strand is complementary to the positive-sense strand and contains an unblocked phosphate at the 5′ end [10]. Two concentric protein shells, the outer capsid and core, comprise the virion particle [1]. Removal of outer capsid proteins during cell entry leads to deposition of a transcriptionally active core particle into the cytoplasm [11–13]. Nascent viral transcripts are extruded from channels at the icosahedral vertices of the core into the cytosol that are translated to make viral proteins [1]. Viral transcripts and newly synthesized viral proteins coalesce and create new cores in a neo-organelle called the viral factory. Viral transcripts are used as a template for synthesis of negative-sense RNAs within newly assembled core particles. Secondary rounds of transcription occur within the viral factories that amplify viral RNA and protein synthesis. Outer capsid proteins are added to the newly formed core particles to produce progeny virions that are released from cells by an unknown mechanism [1].

Transfection of cells with genomic dsRNA alone produces a minimal amount of viral progeny [14]. However, reovirus recovery is markedly increased by transfecting cells with viral ssRNA or

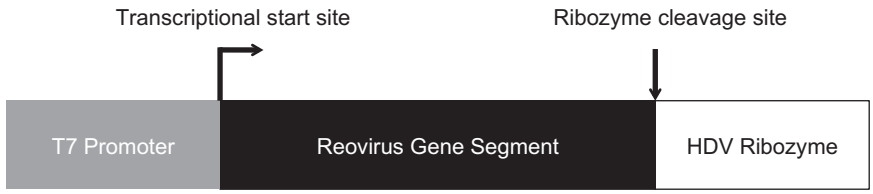

Fig. 1 Schematic of the reovirus T7 transcription cassette. Each reovirus gene segment cDNA is cloned into the plasmid vector downstream of a T7 polymerase promoter sequence and upstream of an HDV ribozyme sequence. The T7 transcriptional start site and HDV ribozyme cleavage site are indicated

dsRNA that was pre-incubated in rabbit reticulocyte lysate to allow translation of viral proteins, and then infecting with an attenuated helper reovirus [14]. Although infectious reovirus can be generated using the helper virus-based system, the technique is cumbersome and inefficient. Moreover, use of the helper virus increases the risk of reassortment between progeny virus and helper virus. However, the ability to rescue virus from ssRNA or melted dsRNA indicated that the positive-sense strand could be used to drive viral replication.

A plasmid-based reverse genetics system for reovirus was developed based on these observations [15]. Single plasmids encoding each of the ten reovirus gene segments were cloned downstream of bacteriophage T7 RNA polymerase promoter (Fig. 1). A hepatitis delta virus (HDV) ribozyme was inserted immediately downstream of the 3′ end. These features are designed to produce RNA transcripts that contain native reovirus 5′ and 3′ termini [16, 17]. The first-generation reovirus plasmid-based reverse genetics system relied on modified vaccinia virus strain DIs (rDIs) to supply T7 polymerase [15, 18]. To recover virus from plasmids, L929 cells were infected with rDIs prior to transfection with plasmids encoding all ten reovirus gene segments. Viable virus was recoverable within 48 h post-transfection [15]. Longer incubation times permitted amplification of rescued virus and yielded higher recovery titers. To increase rescue efficiency, a second-generation system employed baby hamster kidney cells that stably express T7 RNA polymerase (BHK-T7 cells) (Fig. 2) [19]. Use of BHK-T7 cells enhances the efficiency of reovirus recovery by ensuring that T7 RNA polymerase is expressed in every cell that receives plasmids. The second-generation system also uses plasmids that encode multiple reovirus gene segments to further enhance rescue efficiency by reducing the number of plasmids that must be taken up by a single cell. Currently, infectious reovirus can be recovered using as few as four plasmids [19].

Reovirus has long been at the forefront of viral genetics because the segmented genome enables mapping of serotype-specific phenotypic differences to an individual gene [1].

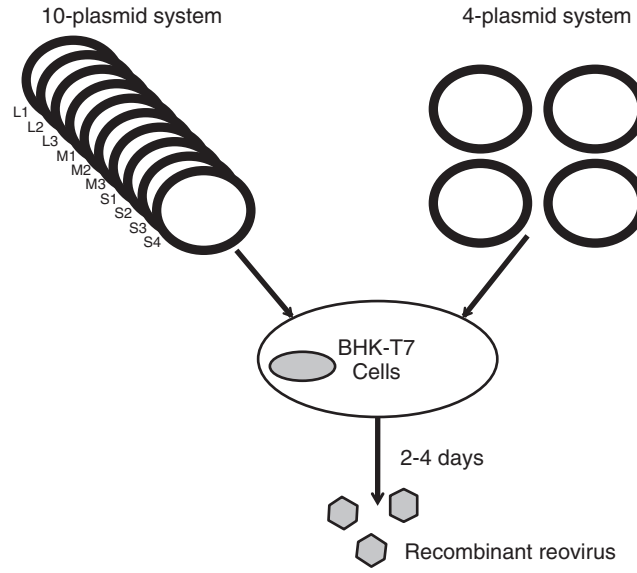

Fig. 2 Reverse genetics for recombinant reovirus rescue. Using the ten- or four-plasmid system, BHK-T7 cells are transfected with plasmids containing reovirus cDNA. The cells are incubated at 37 °C for 2–4 days and then lysed by multiple freeze/thaw cycles to harvest recombinant reovirus

Coinfection of cells with two distinct reovirus serotypes produces reassortant viruses, which are progeny viruses that contain different combinations of gene segments from the parental strains. Panels of reassortant viruses with known genomic content can be tested for the capacity to elicit a specific phenotype. Statistical analysis is employed to determine which gene or genes associate with a particular phenotypic effect. Reassortant reoviruses can be generated by plasmid-based reverse genetics system by blending the desired combination of plasmids. Single-gene reassortant viruses can be produced by individually replacing a gene segment in one genetic background with a single-gene segment from a different reovirus strain (Fig. 3). More genetically complex reassortant panels can be created from pools of viruses that contain multiple gene segments from each parental strain. Gene segments associated with a specific phenotype can be identified using the same analyses applied to traditional reassortant panels.

2 Materials

2.1 Cell Lines and Reagents

All cell culture reagents should be sterile.

1. Baby hamster kidney (BHK-21) cell line that constitutively expresses bacteriophage T7 RNA polymerase (BHK-T7) [20] (*see* **Note 1**).

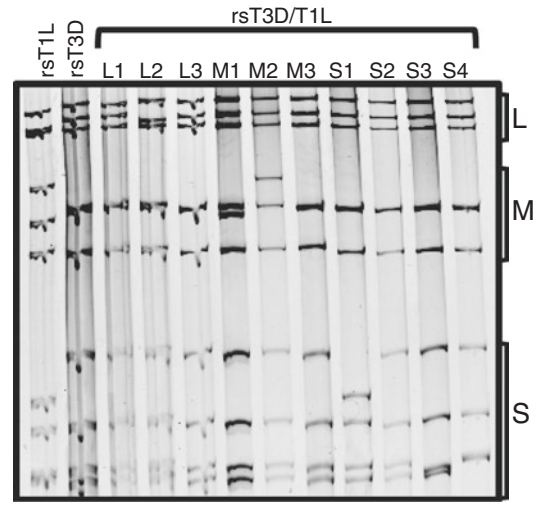

Fig. 3 Electrophoretic analysis of a reovirus single-gene reassortant panel. Purified virions were electrophoresed in a 10% SDS-polyacrylamide gel, followed by ethidium bromide staining (0.5 μg/mL) to visualize viral dsRNA gene segments. Shown are recombinant wild-type strains rsT1L and rsT3D, along with ten single-gene reassortants in which a single-gene segment from T3D was replaced with a gene segment from T1L. The size classes of the large, medium, and small gene segments are indicated as L, M, and S, respectively

2. Spinner-adapted mouse L929 cells.
3. Complete Dulbecco's modified Eagle's MEM (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL of penicillin + 100 μg/mL of streptomycin mixture (Invitrogen), and 250 ng/mL of amphotericin B (Sigma). Store at 4 °C.
4. OPTI-MEM I reduced serum medium (Invitrogen). Store at 4 °C.
5. Complete Joklik's MEM (JMEM) (Sigma) supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin + 100 μg/mL of streptomycin mixture, and 250 ng/mL amphotericin B. Store at 4 °C.
6. Double concentration (2×) Med199 medium (Sigma), incomplete (*see* **Note 2**). Store at 4 °C.
7. Complete 2× Med199 medium supplemented with 5% fetal bovine serum, 4 mM L-glutamine, 200 U/mL penicillin + 200 μg/mL of streptomycin mixture, and 500 ng/mL of amphotericin B. Store at 4 °C.
8. Geneticin® (Invitrogen).
9. 2% Bacto-Agar solution (Fisher Scientific) (*see* **Note 3**).

10. 1% Neutral red solution (*see* **Note 4**).
11. 1× Phosphate-buffered saline (PBS).
12. Tissue culture-treated 60 mm dishes, 6-well plates, and 25 cm^2 flasks (Corning).
13. 65 °C Water bath.
14. TransIT-LT1 transfection reagent (Mirus).

2.2 Reovirus-Encoding Plasmid DNAs

All plasmids that encode reovirus gene segments contain ampicillin resistance genes for selection during growth in bacterial culture [15, 19, 21]. Plasmid DNA purified by maxiprep or midiprep techniques is sufficient for use in rescue reactions.

1. Ten-plasmid system: Individual plasmids encoding single-gene segments from reovirus strains T1L and T3D are designated pT7-L1, pT7-L2, pT7-L3, pT7-M1, pT7-M2, pT7-M3, pT7-S1, pT7-S2, pT7-S3, and pT7-S4.
2. Four-plasmid system: To reduce the number of plasmids utilized for reovirus plasmid-based rescue, cDNAs for multiple gene segments were cloned into single plasmids (*see* **Note 5**).

2.3 Confirmation of Reovirus Genes

1. T3D-L1 and T1L-L3 gene primers to confirm silent mutations [15, 19] (*see* **Note 6**).
2. Gene-specific primers to confirm T1L and T3D sequence of interest (*see* **Note 7**).
3. Thermal cycler.

3 Methods

3.1 Generation of Recombinant Reovirus Using BHK-T7 Cells

1. Culture BHK-T7 cells in complete DMEM growth medium at 37 °C in a humidified atmosphere containing 5% CO_2. The growth medium is supplemented with Geneticin® (1 mg/mL) during alternating passages in culture to maintain selective pressure for the T7 construct. For reovirus rescue, plate tissue culture-treated 60 mm dishes with 3×10^6 cells one day prior to rescue reaction. For rescue, cells are plated without Geneticin® and should be approximately 90% confluent at the time of transfection.
2. For each rescue reaction, pipet 750 μL of OPTI-MEM I into a 1.5 mL microcentrifuge tube. Pipet 53.25 μL of TransIT-LT1 directly into the OPTI-MEM I (*see* **Note 8**). Mix by pipetting gently or vortexing for 2 s. Incubate mixture at room temperature (RT) for 20 min.
3. In a separate 1.5 mL microcentrifuge tube, combine the plasmid DNA. Use additional tubes for two controls: (i) a no-DNA (mock) control and (ii) the desired plasmid mixture

lacking one plasmid (negative control). A total of 17.75 μg of plasmid DNA is used for each rescue (*see* **Note 9**).

4. Add the plasmid mixture directly into the tube containing TransIT-LT1/OPTI-MEM I and mix by pipetting gently or vortexing for 2 s. Incubate mixture at RT for 30 min.
5. Remove medium from tissue culture dish containing attached BHK-T7 cells and replace with 5 mL of complete JMEM.
6. Add the plasmid DNA/TransIT-LT1/OPTI-MEM I mixture to the BHK-T7 cells in a slow, drop-wise manner. Incubate at 37 °C for 1–4 days (*see* **Note 10**).
7. Place the 60 mm dishes at −20 °C. Perform two freeze/thaw cycles to release intracellular virus.
8. Transfer the lysates to an appropriately sized sterile tube and store at −20 °C.

3.2 Recovery and Isolation of Recombinant Reoviruses

Recombinant reovirus is isolated by plaque assay on L929 cells.

1. Culture L929 cells in complete JMEM at 37 °C. One day prior to plaque assay, seed tissue culture-treated 6-well plates with 1×10^6 cells per well (*see* **Note 11**).
2. Perform tenfold serial dilution of lysates using sterile PBS as the diluent.
3. Completely melt 2% agar by microwaving and place in 65 °C water bath until the time of use.
4. Label plates appropriately and decant the plating media. Adsorb 100 μL of each virus dilution to duplicate wells. Incubate at room temperature with rocking every 10–15 min for 1 h.
5. Prepare a 1:1 mixture of complete 2× Med199 media and 2% agar. Overlay each well with 3 mL of the mixture. Incubate at 37 °C.
6. At day 3 post-infection overlay each well with 2 mL of 2× Med199 and 2% agar mixed in a 1:1 ratio. Incubate at 37 °C.
7. At day 6 post-infection, prepare a 1:1 mixture of non-supplemented 2× Med199 and agar. Add 3 mL of 1% neutral red solution per 100 mL of 2× Med199/agar mixture. Overlay each well with 2 mL. Incubate at 37 °C overnight.
8. Invert the plate over a light box and draw a circle around isolated plaques using a sharpie marker.
9. Use a sterile cotton-plugged Pasteur pipet with a rubber bulb attached to collect individual plaques. Expel the air from the rubber bulb and position the pipet tip directly over an isolated plaque. Insert pipet tip through agar overlay until the pipet touches the cell monolayer.

10. Gently rotate the pipet while simultaneously releasing the rubber bulb to retrieve agar and infected cells.
11. Expel plaque contents into 1 mL of sterile PBS in a 1.5 mL microcentrifuge tube. Store at 4 °C for ≥8 h to allow virus to diffuse from the agar plug. This will be used for the propagation of reovirus stocks (*see* **Note 12**).

3.3 Confirmation of Recombinant Reovirus

To confirm the sequence of the virus, extract viral RNA from purified virions and subject to RT-PCR using primers to amplify the gene segments of interest. Analyze purified PCR products by direct sequencing. To confirm that the rescued virus is a recombinant reovirus, amplify and sequence the L3 or L1 gene (*see* **Note 6**).

4 Notes

1. BHK-T7 cells were generated using a T7 RNA polymerase-expressing plasmid encoding a neomycin resistance gene for selection [20].
2. Incomplete 2× Med199 medium is used for staining of the plaque assay for virus recovery.
3. To produce a 2% solution, 10 g Bacto-agar is combined with 500 mL ddH_2O, and then autoclaved for 20 min on a liquid cycle. Store at RT until needed. Melt agar in a microwave prior to use.
4. Mix 5 g neutral red powder with 500 mL ddH_2O and stir overnight. Protect from light by covering beaker in aluminum foil. Filter neutral red solution using a 0.45 μm filter, protected from light. Store at RT in a foil-wrapped bottle.
5. The gene segments for T1 and T3 reovirus are grouped on plasmids as follows.

Virus strain	Gene segment combinations
T1L	L1/M2, L2/M3, L3/S3, M1/S1/S2/S4
T3D	L1/S1, L2/M3, L3/M1, M2/S2/S3/S4

When a reovirus gene is altered via mutation, it is preferable to perform mutagenesis on the single-gene version of the plasmid to minimize off-target changes introduced by polymerase error. In these cases, single-gene-encoding plasmids and multiple-gene segment plasmids can be combined to yield the full complement of ten gene segments.

6. To discriminate between recombinant and nonrecombinant reoviruses, silent mutations were introduced into the plasmids encoding the L3 (C➔T at nucleotide 2059) and L1 (G➔A at

nucleotide 2205) genes from T1L and T3D, respectively [15, 19]. These mutations should be confirmed for all rescued reoviruses.

7. The best practice is to sequence the entire gene segment into which a mutation was inserted.
8. The TransIT-LT1 reagent is used at a ratio of 3 μL TransIT-LT1 per 1 μg plasmid. Be careful not to touch the sides of the microcentrifuge tube with the pipet tip because TransIT-LT1 will stick to the sides of the tube.
9. When using the ten-plasmid system, use the indicated amount of each plasmid.

Plasmid	Quantity (μg)
pT7- L1, pT7-L2, and pT7-L3	2
pT7-M1, pT7-M2, and pT7-M3	1.75
pT7-S1	2
pT7-S2, pT7-S3, and pT7-S4	1.5

When using the four-plasmid system, 4.44 μg of each plasmid is used. If an intermediate number of plasmids are used, divide the 17.75 μg of total plasmid DNA required by the number of plasmids used.

10. rsT1L and rsT3D can be recovered 24 h post-transfection using the four-plasmid system. Peak titers are recovered 48 h post-transfection.
11. Alternatively, plates may be seeded at 2×10^6 cells per well and used the same day. Allow the cells to attach to plate for at least 2 h at 37 °C prior to use.
12. For every virus to be amplified, seed one T25 tissue culture flask with L929 cells at 2×10^6 cells per flask. Seed an additional flask as an uninfected control. Remove the media from each flask, transfer the agar plug in 1 mL of PBS to the flask, and rock gently to coat the cells. Use 1 mL of PBS to mock infect the control flask. Adsorb for 1 h with periodic rocking (10–15-min intervals). Add 5 mL of complete J-MEM and incubate at 37 °C until complete cytopathic effect (CPE) is observed (7–10 days). If CPE is not observed, harvest infected cells when cells in the uninfected flask are dead. Harvest infected cells by performing two freeze/thaw cycles at −20 °C and transfer the lysate to a sterile tube. The first amplification is referred to as passage 1 (P1) stocks. To generate passage 2 (P2) stocks, adsorb a T75 flask with 0.5 mL of the P1 stock. Titer the P2 stocks by plaque assay on L929 cells as described in Subheading 3.2. P2 stocks can be used to generate purified high-titer reovirus stocks [22].

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Chapter 2

Development and Characterization of an Infectious cDNA Clone of Equine Arteritis Virus

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Abstract

Development and characterization of several infectious cDNA clones of equine arteritis virus (EAV) have been described in the literature. Here we describe the assembly of the full-length infectious cDNA clone of the virulent Bucyrus strain (VBS; ATCC VR-796) of EAV in a plasmid vector. This system allows generation of infectious in vitro-transcribed (IVT) RNA from the linearized plasmid that can be transfected or electroporated into mammalian cells to produce infectious recombinant progeny virus. This is an efficient reverse genetics system that allows easy manipulation of EAV genomes to study molecular biology of the virus and pathogenesis of equine viral arteritis.

Key words Equine arteritis virus, EAV, Equine viral arteritis, EVA, Arteriviruses, Infectious cDNA clone, Reverse genetics

1 Introduction

It has long been known that positive-sense viral RNA is infectious and can generate progeny virus following its introduction into cells. Alexander and colleagues first demonstrated the infectivity of poliovirus RNA in HeLa cells [1, 2]. Subsequently, Racaniello and Baltimore developed the first infectious cDNA clone of poliovirus by cloning the full-length RNA genome into a bacterial plasmid vector [3, 4]. The advent of reverse transcription polymerase chain reaction (RT-PCR) technology in the mid-1980s, along with other recombinant DNA techniques, expedited the development of infectious cDNA clones of other RNA viruses [5, 6]. It was subsequently shown in numerous virus systems that in vitro transcripts of cDNA clones, and in some instances the cDNA itself, can initiate a complete productive infectious cycle in susceptible mammalian cells. As a result, genetic manipulation (reverse genetics) of full-length cDNA clones has become the most important tool with which to study the biology, pathogenesis, and virulence determinants of both positive- and negative-stranded RNA viruses.

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Reverse genetic strategies are especially useful for identification and functional characterization of specific viral genes because they demonstrate phenotypic effect(s)/consequences of introducing defined nucleotide change(s) to the gene of interest.

EAV is included within the order *Nidovirales,* and it is the prototype virus of the genus *Arterivirus*, family *Arteriviridae*. Similar to other positive-stranded RNA viruses, the genomes of Arteriviruses are infectious to cells [7, 8]. The first full-length infectious cDNA clone of EAV was developed in 1996 by cloning 12 fragments from a cDNA library spanning the entire genome of a highly cell culture-adapted laboratory strain of EAV downstream of the T7 RNA polymerase promoter in the pUC18 plasmid vector (pEAV030 [GenBank accession number Y07862]) [9]. This was also the first full-length infectious cDNA clone constructed from a member of the order *Nidovirales*. A second infectious cDNA clone of a very similar, highly cell culture-adapted laboratory strain of EAV was described soon thereafter [10–12]. Subsequently, we developed two infectious cDNA clones of EAV: the first from the highly virulent, horse-adapted virulent Bucyrus strain (VBS) of EAV (pEAVrVBS [DQ846751]) [13] and the other from the MLV vaccine strain of EAV (ARVAC®, Zoetis, Kalamazoo, MI, USA, pEAVrMLV [FJ798195]) [14] that was originally developed by extended cell culture passage of the VBS virus.

Here we describe the assembly of the full-length infectious cDNA clone of the virulent Bucyrus strain (VBS; ATCC VR-796) of EAV in the pTRSB plasmid under the control of T7 RNA promoter. The EAV genome is in vitro transcribed (IVT) into RNA using the T7 RNA-dependent RNA polymerase enzyme. At the 3′-end, a 20 bp poly (A) tail is incorporated downstream of the EAV genome followed by a unique restriction site (Xho-I) for linearization of the plasmid to generate runoff IVT RNA. For cloning purposes, another unique restriction enzyme site (Xba-I) is incorporated upstream of the 5′-end of the T7 promoter. This system allows generation of infectious IVT RNA from the linearized plasmid for subsequent electroporation into a mammalian cell line to generate infectious progeny virus.

2 Materials

2.1 Assembly of the Infectious cDNA Clone

1. Plasmid and *E. coli* strain.
   (a) The pTRSB plasmid is available upon request from the authors of this chapter. It carries ampicillin-resistant gene for selection of recombinant clones.
   (b) *E. coli* DH5α™ competent cells: These bacterial cells can be either purchased from Life Technologies (MAX Efficiency®

DH5α™ Competent Cells) or prepared in the laboratory following the protocol described in Subheading 3.6.

2. Culture medium for *E. coli*.
   (a) LB medium (Luria-Bertani medium; 1 L): Deionized water 1000 mL, Bacto-tryptone 10 g, Bacto-yeast extract 5 g, and NaCl 10 g. Stir until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Sterilize by autoclaving for 20 min on liquid cycle.
   (b) LB agar plates: Prepare LB medium according to the above recipe. Just before autoclaving, add 15 g of Bacto agar/1000 mL of LB medium. Sterilize by autoclaving for 20 min on liquid cycle. After the medium is removed from the autoclave, swirl it gently to distribute the melted agar throughout the solution. Allow the medium to cool to 45–50 °C before adding antibiotics (ampicillin 50 μg/mL). To avoid air bubbles, mix the medium by swirling. Pour 20–25 mL of medium into a petri dish (90 mm). After medium has solidified completely, invert the plates, wrap in aluminum foil, and store them at 4 °C until needed. The plates should be removed from storage 1–2 h before they are used in order to allow them to dry.
   (c) LB freezing buffer: 40% (v/v) glycerol in LB medium. Sterilize by passing it through a 0.45 μm disposable filter.
   (d) SOB medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, and 2.5 mM KCl. Adjust the pH to 7.0 with 5 N NaOH and sterilize by autoclaving on liquid cycle (*see* **Note 1**).
   (e) SOC medium: SOB medium containing 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose. After autoclaving the SOB medium, cool to 45 °C and add the $MgCl_2$, $MgSO_4$, and glucose from filter-sterilized 1 M stock solutions.
3. Media and solutions for preparing competent *E. coli* cells.
   (a) Glucose-supplemented LB medium (500 mL): Bacto-tryptone 5.0 g, Bacto-yeast extract 2.5 g, NaCl 2.5 g, and glucose 0.5 g. Bring the volume to 500 mL with distilled water. Autoclave for 30 min on liquid cycle. Store at 4 °C.
   (b) Glycerol 100 mL: Autoclave for 30 min on liquid cycle. Store at 4 °C.
   (c) 1 M $MgCl_2$ stock (100 mL): $MgCl_2 \cdot 6H_2O$ (FW 203.30) 20.33 g in 100 mL of distilled water. Autoclave for 30 min on liquid cycle. Store at room temperature.
   (d) 1 M $CaCl_2$ stock (100 mL): $CaCl_2 \cdot 2H_2O$ (FW 47.02) 14.70 g in 100 mL of distilled water. Autoclave for 30 min on liquid cycle. Store at room temperature.

(e) Prepare working solutions: 0.1 M $MgCl_2$ working solution (100 mL) and 0.1 M $CaCl_2$ working solution (100 mL; *see* **Note 2**).

4. Special buffers and solutions.

   Ampicillin stock (50 mg/mL): Dissolve solid ampicillin in sterile water to a final concentration of 50 mg/mL and filter through a 0.45 μm filter. Store the solution in the dark at −20 °C.

5. Enzymes and buffers.

   Restriction endonucleases, T4 DNA ligase, high-fidelity DNA polymerase, and reverse transcriptase. These enzymes can be purchased from various commercial sources (*see* **Note 3**). Use the buffer supplied with the enzyme by the manufacturer.

6. Other Molecular Biology Kits, Reagents, and Other Materials

   (a) QIAamp Viral RNA Mini Kit (Qiagen).

   (b) QIAprep Spin Miniprep Kit (Qiagen).

   (c) QIAgen Plasmid Maxi Kit (Qiagen).

   (d) QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc.).

   (e) MagMAX™-96 Viral RNA Isolation Kit (Life Technologies).

   (f) Magnetic-Ring Stand (Life Technologies) for 96-well plates.

   (g) U-bottom plates and lids (Evergreen Scientific).

   (h) Orbital shaker (Multi-Microplate Genie, Scientific Industries Inc.).

   (i) Proteinase K (Life Technologies).

   (j) 100% Ethanol, molecular biology grade (Sigma).

   (k) 100% Isopropanol, molecular biology grade (Sigma).

   (l) Single-channel and multichannel pipets.

   (m) RNase-free filter tips (aerosol-resistant tips).

   (n) Protective gear: lab coat, gloves, and goggles.

   (o) RNaseZap Solution (Life Technologies).

   (p) Ice buckets and trays.

   (q) Sterile autoclave bottles (250 mL) or tubes.

   (r) RNase/DNase-free microcentrifuge tubes.

   (s) Sterile screw-cap tubes.

   (t) 0.45 μm Filters.

   (u) Sterile 15 and 50 mL conical tubes.

   (v) Falcon 15 mL polypropylene tubes.

   (w) Amicon Ultra® concentration columns (EMD Millipore).

2.2 Rescue of Recombinant Virus

1. Cells.
   (a) Equine endothelial cells (EECs) are available upon request from the corresponding author of this chapter.
   (b) Baby hamster kidney cells (BHK-21; ATCC, CCL-10, Manassas, VA, USA).
   (c) Rabbit kidney cells (RK-13; ATCC, CCL-37, Manassas, VA, USA).
2. Cell culture medium.
   (a) The EECs are maintained in Dulbecco's modified essential medium (Mediatech, Manassas, VA) with sodium pyruvate, 10% fetal bovine serum (FBS; HyClone Laboratories, Inc.), 100 U/mL penicillin-100 μg/mL streptomycin, and 2 mM L-glutamine (Mediatech).
   (b) BHK-21 and RK-13 cells are maintained in Eagle's minimum essential medium (EMEM; Mediatech) supplemented with 10% ferritin-supplemented bovine calf serum (HyClone Laboratories, Inc), and 100 U/mL penicillin-100 μg/mL streptomycin (Gibco).
   (c) Trypsin-EDTA solution: 0.25% (w/v) trypsin, 0.02% (w/v) EDTA.
3. In Vitro transcription reagents.
   In vitro-transcribed (IVT) RNA synthesis from linearized plasmid can be performed either with a commercial kit (mMESSAGE mMACHINE® kit (Life Technologies)) or in-house assembly of the reaction using individually purchased reagents (m^7G[5′]PPP[5′]G RNA cap structure analogue (New England BioLabs)), recombinant RNasin® ribonuclease inhibitor [40 U/μL], 5 μL of rATP, rCTP, rGTP, and rUTP [10 mM mix], 2.5 μL of 100 mM DTT, 2.5 μL of T7 RNA polymerase, and 1× transcription buffer (Promega).
4. Miscellaneous molecular biology-grade reagents.
   Agarose, 10% SDS, 0.5 M EDTA (pH = 8.0), TE buffer (pH = 7.2), and gel-loading buffer (6×).
5. Special equipment.
   Gene Pulser Xcell™ Electroporation Systems (Bio-Rad) or BTX electroporation system (Harvard Apparatus) fitted with electrodes spaced 0.4 cm.

3 Methods

3.1 General Strategy for the Assembly of Full-Length EAV VBS cDNA Clone

The basic strategy for the generation of EAV infectious cDNA clone is described using the EAV VBS (GenBank accession number DQ846751) as a model. The assembly of the full-length infectious cDNA clone of EAV VBS is facilitated by the construction of two

intermediate shuttle plasmids containing the 5′-end (nucleotide numbers 1–4629) and 3′-end (4192–12,704 plus engineered poly[A] tail) of the genome under the control of the T7 promoter (Fig. 1). The two cloned EAV fragments overlap and have a

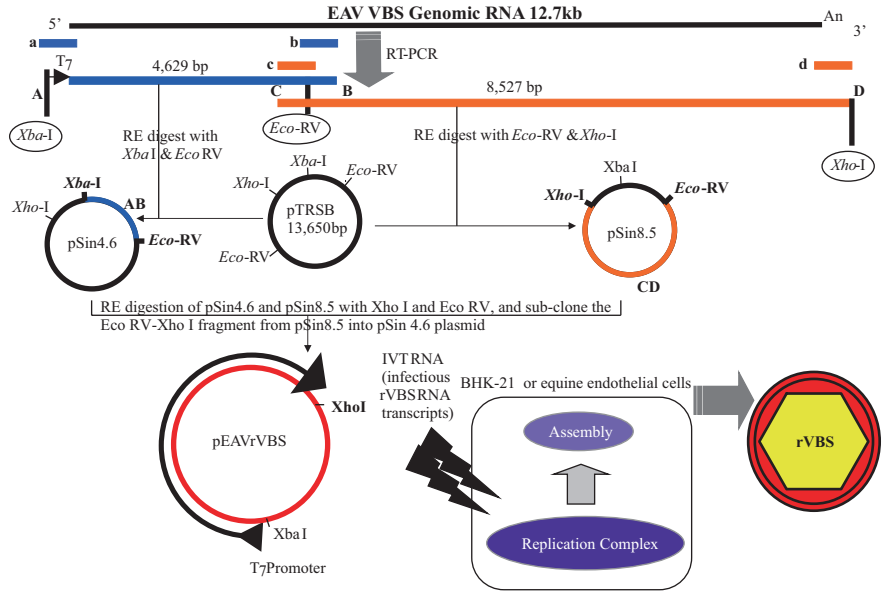

Fig. 1 Construction of the full-length cDNA clone of the virulent Bucyrus strain of EAV. Genome organization and the unique *Eco*-RV site in ORF1a are shown at the top. The four synthetic oligonucleotide primers that were used for RT-PCR amplification of the complete genome in two segments are identified by (*a*) (EXT5′T7P: 5′ CTA GAT CC***T CTA G***AT TAA TAC GAC TCA CTA TAG CTC GAA GTG TGT ATG GTG CCA TAT ACG GC 3′ [5′-end restriction site Xba I italicized and the T7 promoter underlined]), (*b*) (4556N: AAT GTT GCA GTG AGA CTC TCC TGG G 3′), (*c*) (4192P: 5′ TCC ATG CGC TTG TGC TTG TTC CAT C 3′), and (*d*) (12,687TN: 5′ TTT TT***C TCG AG***T TTT TTT TTT TTT TTT TTT TGG TTC CTG GGT GGC TAA TAA CTA C 3′ [5′-end restriction site XhoI italicized and poly(T) tail underlined]). Two overlapping cDNA fragments (*AB* and *CD*) represented by thick lines were amplified from genomic RNA through RT-PCR to cover the entire EAV genome. The 5′ PCR segment was 4629 base pairs and had a 5′ *Xba*-I site as well as a T7 promoter sequence, and a natural unique *Eco*-RV site at the 3′-end. The 3′-PCR segment was 8527 base pairs and had a 3′-poly (A) tail (20 nucleotides) followed by a *Xho*-I site, and a natural *Eco*-RV site at the 5′-end. The PCR segment containing the 5′-end of the genome was cut with *Xba*-I and *Eco*-RV and cloned into pTRSB vector that has also been cut with the same restriction enzymes. This recombinant plasmid containing the 5′-end of the VBS genome was named pSin4.6. The PCR segment containing the 3′-end of the genome was cut with *Eco*-RV and *Xho*-I and cloned into pTRSB vector which has also been cut with the same restriction enzymes. This recombinant plasmid containing the 3′-end of the VBS genome was named pSin8.5. These plasmids were transformed into *E. coli* (HB101; Invitrogen), and the authenticity of each insert was confirmed by restriction enzyme digestion, and sequencing. The pSin8.5 recombinant plasmid was digested with *Eco*-RV and *Xho*-I restriction enzymes and the restriction fragment containing the 3′-end was gel-purified. This fragment then was subcloned into pSin4.6 plasmid that had also been cut with the same restriction enzymes. This new recombinant plasmid contained a complete copy of the viral cDNA downstream of T7 promoter. This recombinant plasmid was transformed into *E. coli* (DH5α; Invitrogen), and authenticity of the insert was confirmed by restriction enzyme digestion. One full-length plasmid clone sequence was determined by sequencing of both strands using automated sequencing. After confirming the authenticity of the full-length sequence this plasmid was identified as the pEAVrVBS, amplified in DH5α *E. coli*, and purified using a QIAgen Plasmid Maxi Kit (Qiagen)

common restriction site (e.g., ECoRV) to be assembled into a full-length infectious cDNA clone of the virus (*see* **Note 4**). The full-length cDNA clone has the unique restriction sites selected in the first step, a T7 promoter sequence at the 5′-end followed by the cDNA sequence of the EAV VBS strain, and a 20 nt synthetic poly(A) at the 3′-end which is followed by a unique restriction site to be used for the linearization of the plasmid to generate infectious IVT RNA. All these elements have to be precisely assembled to produce synthetic IVT RNA bearing authentic 5′- and 3′-end of the viral genome (Fig. 1; *see* **Note 5**).

1. Cloning strategy.

   The first step in developing an infectious cDNA clone is the careful design of the RT-PCR amplification and cloning of the full-length cDNA copy of desired EAV strain into a bacterial plasmid under a promoter (e.g., T7, SP6, or CMV; in this protocol, we describe the use of T7 promoter). Ideally, the master sequence of the EAV strain that is to be cloned should be obtained by sequencing the entire genome using high-fidelity reverse transcriptase and DNA polymerase enzymes. The primers for PCR amplification and sequencing could be designed for the conserved regions of the EAV sequences available in GenBank. Even though the sequence of the virus strain that is intended to be cloned is available in GenBank, it is a good idea to sequence the RNA from virus stock that is available in the laboratory. The entire genome sequence including the 5′- and 3′-end of the genome should be determined using 5′- and 3′-RACE technology. This will provide the master sequence of the virus available in the laboratory for the given strain, and help to determine whether it is identical to the published sequence available in GenBank. Once you confirm the sequence of your strain, that sequence should be used to design cloning strategy. Sequence analysis and primer design could be performed using Vector NTI (Life Technologies) or Genius 7.0.6 software (Biomatters Ltd.)

2. Selection of restriction endonuclease sites in the viral genome.

   The next step in the assembly of the full-length cDNA clone is the selection of appropriate restriction endonuclease sites in the viral genome. These restriction sites must be present in the cloning vector (e.g., pTRSB plasmid). In the case of EAV VBS the restriction sites selected are EcoRV (natural site at nucleotide position 4228), Xba I (an engineered site upstream of T7 promoter), and XhoI (an engineered site downstream of poly(A) tail). Synthetic primers are designed according to the published sequences of EAV VBS, GenBank accession # DQ846750), and used for RT-PCR amplification of the EAV VBS genome in two overlapping fragments. Two synthetic oligonucleotide primers A and B (Fig. 1) are used to RT-PCR amplify the 5′-end of the EAV genome. The

positive-sense primer A (62 nt's) consists of 30 nucleotides of the extreme 5′-end of the EAV VBS genome with an engineered overhanging Xba I site and a T7 promoter sequence. The negative-sense primer B is 309 nucleotides downstream of the natural EcoRV site (at nt 4228). The PCR segment is 4629 base pairs and has a 5′-Xba I site as well as a T7 promoter sequence, and a natural EcoRV site at the 3′-end. Two synthetic oligonucleotide primers C and D (Fig. 1) are used for the RT-PCR amplification of the 3′-end of the EAV genome. The negative-sense primer D is complementary to the extreme 3′-end of the genome (last 24 nt of the EAV genome) and has an overhanging poly(T) tail (20 nt) and a XhoI restriction enzyme site. The positive-sense primer C is 33 nucleotides upstream of the natural EcoRV site (at nt 4228). The PCR segment is 8527 base pairs (Fig. 1) and has a 3′-poly(A) tail followed by a XhoI site, and a natural EcoRV site at the 5′-end.

3.2 RT-PCR Amplification of EAV VBS Genome into Two Overlapping Fragments

1. cDNA synthesis, PCR amplification, and cloning of EAV VBS
   (a) Isolate genomic viral RNA from the tissue culture fluid containing EAV VBS strain using QIAamp viral RNA purification columns (QIAamp® viral RNA mini kit, Qiagen) according to the manufacturer's instructions. Alternatively, genomic viral RNA could also be isolated using MagMAX™-96 Viral RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions.
   (b) The first-strand cDNA is synthesized using the Superscript™ II RNase H-reverse transcriptase (Life Technologies [formerly Invitrogen]) according to the manufacturer's instructions. Two first-strand cDNAs are synthesized using primers B and D, respectively.
   (c) Amplification of two long PCR fragments covering the EAV VBS entire genome is carried out according to the manufacturer's instructions with the Expand™ Long Template PCR System (Roche, Indianapolis, IN, USA) using the aforementioned primer pairs A and B, and C and D (*see* **Note 6**). This system utilizes a unique enzyme mixture containing Taq DNA polymerase (5′–3′ polymerase activity) and Pwo DNA polymerase (3′–5′ proofreading ability). The PCR products are concentrated (Amicon Ultra®, EMD Millipore, Billerica, MA, USA) and agarose gel purified using a commercial kit (QIAquick Gel Extraction Kit, Qiagen; *see* **Note 7**).

3.3 Assembly of the Full-Length Infectious cDNA Clone of EAV

1. Construction of intermediate plasmids containing the 5′- and 3′-EAV sequences.

   The PCR segment containing the 5′-end of the genome is digested with XbaI and EcoRV and cloned into the pTRSB vector, which also has been cut with the same restriction enzymes.

This recombinant plasmid containing the 5′-end of the EAV VBS genome is named pSin4.6. The PCR segment containing the 3′-end of the genome is cut with EcoRV and XhoI and cloned into pTRSB vector, which also has been cut with the same restriction enzymes. This recombinant plasmid containing the 3′-end of the EAV VBS genome is named pSin8.5. These plasmids are transformed into *E. coli* (DH5α or HB101; Life Technologies); the authenticity of each insert was confirmed by restriction enzyme digestion, and sequencing of multiple plasmids. Two plasmids with authentic 5′- and 3′-sequences of EAV are selected to be assembled into the full-length genome.

2. Amalgamation of 5′- and 3′-sequences to generate the full-length infectious cDNA clone of EAV.

   The 3′-end of the EAV VBS genome from pSin8.5 plasmid is subcloned into the pSin4.6 plasmid. Briefly, the pSin8.5 recombinant plasmid is digested with EcoRV and XhoI restriction enzymes and the restriction fragment containing the 3′-end of the EAV VBS is gel-purified. This fragment is then subcloned into the pSin4.6 plasmid that has also been cut with the same restriction enzymes. This new recombinant plasmid contains the complete copy of the viral cDNA downstream of a T7 promoter. This plasmid is transformed into *E. coli* (DH5α; Life Technologies) and authenticity of the insert is confirmed by restriction enzyme digestion. One full-length plasmid clone is selected for nucleotide analysis, and the complete sequence is determined from both strands using automated sequencing. Recombinant plasmid DNA containing the authentic EAV genome (cDNA copy) is stored at −20 °C.

3.4 Transformation of Competent E. coli and Purification of Plasmid DNA

1. Remove DH5α cells from −80 °C and thaw on ice.
2. Gently mix DH5α cells with pipet and aliquot 100 μL into chilled polypropylene tubes (Falcon).
3. Dilute 1 μL of plasmid DNA into 19 μL sterile nuclease-free water. Add 2.5 μL of 1:20 diluted plasmid DNA into 100 μL of bacterial cells.
4. Incubate cells on ice for 30 min.
5. Preheat SOC medium in a 42 °C water bath for use in **step 8** below.
6. Heat-shock cells for 45 s in a 42 °C water bath (*see* **Note 8**).
7. Place on ice for 2 min.
8. Add 0.9 mL of preheated SOC medium.
9. Incubate the tubes at 37 °C with 1 h shaking at 240 rpm.
10. After incubation at 37 °C for 1 h with shaking, the bacteria and medium are transferred to a microcentrifuge tube, and centrifuged at 4000 × *g* for 3 min. Decant the supernatant and leave about 100 μL supernatant in the tube. Resuspend the

cells and plate 70 μL of cells onto one LB agar plate with 100 μg/mL ampicillin. Plate the remaining 30 μL of cells onto another LB agar plate with 100 μg/mL ampicillin. Incubate the plates overnight at 37 °C.

11. The amplification and isolation of plasmid DNA are performed using standard procedures described for conventional plasmids. Select individual bacterial colonies (2–6 colonies) for screening (*see* **Note 9**). Inoculate 2 mL of LB broth containing ampicillin with an individual bacterial colony and incubate at 37 °C overnight in a shaker incubator (240 rpm). Purify the plasmid DNA using QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Authenticity of the plasmid is confirmed by restriction digestion and sequencing. Prepare large-scale working plasmid stock by inoculating fresh LB broth medium (250 mL) containing ampicillin. Briefly dilute 0.1 mL of the culture into 250 mL of selective LB medium pre-warmed to 37 °C and grow the cells with vigorous shaking (250 rpm) in a 1 l flask at 37 °C for 12–16 h (overnight) until an OD value of 1.2–1.5 at 550 nm is reached. This cell density typically corresponds to the transition from a logarithmic to a stationary growth phase. Harvest the bacterial cells by centrifugation at 6000 × *g* for 15 min at 4 °C and purify the plasmid DNA with the QIAgen Plasmid Maxi Kit.

3.5 Storage of Bacterial Cultures

1. Mix 0.5 mL of LB freezing medium with 0.5 mL of an overnight bacterial culture in a cryotube with a screw cap (*see* **Note 10**).
2. Vortex the culture to ensure that the glycerol is evenly dispersed, freeze in ethanol-dry ice, and transfer to −80 °C for long-term storage.
3. Alternatively, a bacterial colony can be stored directly from the agar plate without being grown in a liquid medium. Using a sterile pipet tip, scrape the bacteria from the agar plate, and resuspend the cells into 200 μL of LB medium in a cryotube with a screw cap. Add an equal volume of LB freezing medium, vortex the mixture, and freeze the bacteria as described above.
4. To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculating needle and then immediately streak the bacteria that adhere to the needle onto the surface of an LB agar plate containing appropriate antibiotics (e.g., ampicillin; *see* **Note 11**). Incubate the plate at 37 °C overnight. Return the original frozen culture to storage at −80 °C.

3.6 Preparation of DH5α Competent Cells for Electroporation

Here we describe the protocol for preparing electrocompetent *E. coli* DH5α cells from 500 mL of bacterial culture. All the steps of this protocol should be carried out under sterile conditions.

1. Streak out *E. coli* (DH5α, HB101, or other strain) on an LB agar plate (without antibiotics) and incubate overnight at 37 °C.

2. The next day, pick a single bacterial colony and grow in 2 mL of LB medium (without antibiotics), with vigorous shaking (250 rpm) at 37 °C overnight. Take 0.5 mL of the prepared LB medium to use as a blank for the OD. Transfer overnight bacterial prep to a 2 L flask containing 500 mL of glucose-supplemented LB medium. Incubate at 37 °C with vigorous shaking (250 rpm). During this time, chill all solutions, centrifuge bottles and tubes on ice, turn on spectrophotometer, and set wavelength at 550 nm. After several hours (2.5–3 h), remove 0.5 mL of bacteria using a sterile pipet and check OD550 (use LB/glucose as blank). Continue to check the culture until the OD550 reaches 0.5–0.7 (bacteria double about every 20 min; *see* **Note 12**). As soon as the correct OD is achieved, immediately transfer the culture flask from the shaker to an ice/water bath. Swirl the culture flask occasionally for 5–20 min to ensure that cooling occurs uniformly. From this point on, it is crucial that the temperature of the bacteria does not rise above 4 °C.
3. Pour the bacteria into two chilled (ice-cold) 500 mL centrifuge bottles and spin at 6000 × *g* (6000 rpm in a Sorvall GS3 rotor) for 15–20 min at 4 °C.
4. Carefully decant the supernatant and place bottle with bacteria pellet on ice. Resuspend the pellet in 10 mL of ice-cold 0.1 M $MgCl_2$ using sterile 10 mL pipet. Once resuspended, add the remaining 90 mL of chilled 0.1 M $MgCl_2$. Set on ice for 5 min and spin at 6000 × *g* (6000 rpm in a Sorvall GS3 rotor) for 20 min at 4 °C.
5. While spinning, transfer 8.6 mL of 0.1 M $CaCl_2$ to a 15 mL conical tube. Add 1.4 mL glycerol. Mix well and let sit on ice.
6. Carefully decant the supernatant and place the bottle on ice. Resuspend in 10 mL of ice-cold 0.1 M $CaCl_2$ using sterile 10 mL pipet. Once resuspended, add the remaining 81.4 mL of ice-cold 0.1 M $CaCl_2$. Set on ice for 5 min and spin at 6000 × *g* (6000 rpm in a Sorvall GS3 rotor) for 20 min at 4 °C.
7. Decant supernatant well and place bottle on ice. Resuspend the bacteria in a chilled solution of 8.6 mL 0.1 M $CaCl_2$ containing 1.4 mL glycerol. Mix well and transfer 0.25 mL aliquots to microcentrifuge or screw-cap tubes (approximately 40 tubes) that have been placed in dry ice-methanol bath or drop tubes directly into liquid nitrogen container for quick freeze. Store bacteria at −80 °C until use. Once thawed, the cells should not be frozen again.

3.7 Linearization and Purification of the Plasmid DNA

1. Linearize approximately 10 μg of plasmid DNA per restriction digestion reaction. An example of restriction digestion reaction is given below.

Plasmid DNA	32.0 μL[a]
XhoI	4.0 μL
Buffer (10×)	4.0 μL
Sterile nuclease-free water	To 40.0 μL final volume[b]
Incubate the reaction tube at 37 °C for 2–4 h	

[a]Plasmid volume depends on the DNA concentration
[b]Reaction volume can be adjusted to 40 μL using nuclease-free water

2. Run 1 μL digested plasmid DNA on 1% agarose gel to make sure that the plasmid DNA is linearized. Use an appropriate DNA molecular weight marker on the gel (e.g., 1 kb DNA ladder, Life Technologies [formerly Invitrogen]).

Digested plasmid DNA	1.0 μL
6× loading buffer	2.0 μL
Sterile nuclease-free water	To 12.0 μL final volume

3. Add 1 μL of 20 mg/mL proteinase K, bring the volume to 100 μL with nuclease-free water, and incubate at 37 °C for 30 min.
4. Perform phenol:chloroform extraction twice and precipitate the DNA with 100% ethanol as follows:
   (a) Add 100 μL of phenol:chloroform:isoamyl alcohol (25:24:1) and mix until an emulsion is formed.
   (b) Centrifuge at 16,000 × *g* (13,000 rpm) for 4 min at room temperature.
   (c) Remove 90 μL of the upper aqueous phase and transfer into a new tube.
   (d) Add 90 μL of phenol:chloroform:isoamyl alcohol (25:24:1) and repeat the extraction.
   (e) Remove 80 μL of the upper aqueous phase and transfer to a new tube.
   (f) Add 250 μL of 100 or 96% ethanol and 8 μL of 3 M sodium acetate to the DNA sample.
   (g) Mix and then centrifuge at 13,000 × *g* for 10 min.
   (h) Wash with 70 μL of 70% ethanol.
   (i) Spin at 13,000 × *g* for 4 min.
   (j) Aspirate the 70% ethanol and dry on the bench for 4–5 min.

5. Resuspend the pellet in 16 μL of nuclease-free water and run 1 μL on 1% agarose gel.
6. Store the linearized plasmid at −20 °C until further use.

3.8 In Vitro Transcription of Linearized Plasmid DNA

1. The transcription of cDNA to RNA is carried out with T7 RNA polymerase (MEGAscript kit; Life Technologies). The reaction is performed according to the manufacturer's instructions. The recombinant RNA produced will be capped and have a poly(A) tail; thus when it is transfected into the cells, it will be treated as messenger RNA.
2. Alternatively, the in vitro transcription reaction can be set up by combining various commercial reagents purchased individually.

Linear plasmid DNA (1 μg)	15.0 μL
rNTPs (10 mM each)	5.0 μL
BSA 1 mg/mL	5.0 μL
100 mM DTT	2.5 μL
5× Transcription buffer	10.0 μL
Cap analogue	5.0 μL
RNase inhibitor guard	2.5 μL
T7 RNA polymerase	2.5 μL
Nuclease-free water	To 50.0 μL final volume
Incubate at 37 °C for 1 h	

3. Run 2 μL of in vitro-transcribed RNA on a 0.8% agarose gel.

TE buffer (pH = 7.2)	8.0 μL
10% SDS	1.0 μL
0.5 M EDTA (pH = 8.0)	0.25 μL
RNA	2.0 μL
Place mix at 70 °C for 2 min and then place on ice for 2 min	
Add 2.25 μL of 6× gel-loading buffer	

3.9 Transfection of Mammalian Cells with IVT RNA and Rescue of the Recombinant Virus

Infectious virus is recovered by transfection of susceptible mammalian (equine endothelial or BHK-21) cells with the IVT RNA derived from the full-length cDNA clone. The following protocol is indicated for a 35 mm diameter dish and can be scaled up or down if desired.

1. Preparation of Mammalian Cells for Electroporation of IVT RNA

(a) Split cells (EECs or BHK-21 cells) on day before to be 90–95% confluent by next day. As rule of thumb, propagate cells in 150 cm^2 flasks, and usually this will give enough cells to perform 3–4 electroporations per flask of cells (2.5×10^7; *see* **Note 13**).

(b) Treat cells with trypsin following standard laboratory protocol.

(c) After cells slough off, add 10 mL MEM containing FBS to inactivate residual trypsin.

(d) Use a pipet with a wide bore to transfer cells to a 50 mL conical centrifuge tube. Place on ice immediately.

(e) Spin cells at 4 °C, 300 × *g* (700–800 rpm), for 6 min. Place cells back on ice.

(f) Remove medium with a pipet and add 25 mL sterile ice-cold PBS (pH = 7.4). Resuspend the cell pellet by gently shaking the tube. Cells also can be resuspended by using a wide-bore pipet.

(g) Repeat step (e).

(h) Remove PBS as before and again wash by the addition of 25 mL ice-cold PBS. At this time, take a small sample of cells for counting (i.e., make a 1:20 dilution by adding 50 μL of cells into 950 μL of PBS for counting).

(i) Repeat step (e). While cells are spinning, conduct a cell count with a hemocytometer or an automated cell counter.

(j) After cells have been spun down, place them back on ice and remove PBS with a pipet. Resuspend cell pellet in ice-cold PBS to a final concentration of 1×10^7 cells/mL using the count.

2. Electroporation of Mammalian Cells with IVT RNA.

(a) Set the electroporator to desired voltage (*see* **Note 14**). Gene Pulser (Bio-Rad, Hercules, CA, USA): 1500 V, capacitance at 25 μF, and resistance at infinity Ohms or BTX 600 (Harvard Apparatus, Holliston, MA): 260 V, capacitance at 950 μF, and 13 Ω.

(b) Place 10 μL of freshly thawed transcription mix (~10–20 μg IVT RNA) into each electroporation cuvette (0.2 cm, Bio-Rad, Hercules, CA, USA, or 0.4 cm, BTX, Harvard Apparatus, Holliston, MA, USA) to be used (*see* **Note 15**).

(c) Place 500 μL of cells (5×10^6) into each cuvette. Addition of cells will mix with the IVT RNA. Do not mix the cells and IVT RNA by inverting the cuvette because this will generate bubbles.

(d) Place the cuvette into the cuvette holder and pulse once (BTX) or twice (Gene Pulser; push the buttons until you hear a beep, then immediately push them again until you hear the second beep [time constant reading should appear within the range of 7.0–7.4]).

(e) After electroporation is complete, set cells aside at room temperature for a 10-min "recovery period."

(f) After the recovery period is complete, transfer cells from the cuvette with a Pasteur pipet into 10 mL of cell culture medium in a 15 mL conical tube at room temperature. For immunofluorescence staining, transfer approximately 150 μL of cells into a chamber slide. Then transfer the remaining cells and medium into a single 100 mm petri dish. Sometimes two electroporations can be combined into one petri dish.

3. Rescue and Characterization of Recombinant Virus

(a) Examin transfected cells for expression of EAV nonstructural protein-1 (e.g., nsp-1) and/or structural proteins (e.g., GP5 and N) 12–18 h post-transfection by indirect immunofluorescence staining using protein-specific monoclonal antibodies. Immunofluorescence staining will confirm the infectivity of the transfected RNA.

(b) Incubate the petri dish at 37 °C for 3–5 days until a clear cytopathic effect is observed. Three to five days after transfection (or as soon as the monolayers show significant cytopathic effect), the medium is collected by centrifugation for 10 min at 2000 × *g*. The supernatant will contain the new virus (P0) produced by infectious recombinant RNA. Small aliquots (0.5–1.0 mL) of supernatant are stored at −80 °C.

(c) Analyze the presence of recombinant virus in the supernatant by titration.

(d) Analyze the genotypic and phenotypic properties of the recovered virus (*see* **Note 16**).

3.10 Further Manipulation of the Infectious cDNA Clone Using Site-Directed Mutagenesis

Genetic manipulation of full-length cDNA clones using reverse genetics has become an important and widely used tool to study the biology, pathogenesis, neutralization, and virulence determinants of EAV. Reverse genetic manipulation of EAV infectious cDNA clones can be successfully performed using QuikChange II XL Site-Directed Mutagenesis Kit (Cat # 200522, Agilent Technologies Inc., Santa Clara, CA, USA [formerly Stratagene]). The mutagenic (e.g., mutations, insertions, or deletions) oligonucleotide primers used in this protocol are designed individually following the guidelines provided by the manufacturer.

4 Notes

1. To avoid arcing during electroporation, SOB medium and PBS used in this protocol should be prepared free of Mg^{2+}.
2. Stock solutions can be sterilized by autoclaving and stored at room temperature. 0.1 M solutions should be prepared fresh.
3. It is important to use high-fidelity enzymes for RT-PCR amplification. This will reduce the inadvertent introduction of nucleotide changes during RT-PCR amplification.
4. After each cloning step, the PCR-amplified fragments and cloning junctions have to be sequenced to determine that no undesired mutations are introduced.
5. Recently, we have described the in silico design and de novo synthesis of a full-length infectious cDNA clone of the horse-adapted virulent Bucyrus strain (VBS) of EAV encoding mCherry [15]. This de novo nucleotide synthesis technology facilitated innovative viral vector design without the tedium and risks posed by more complicated conventional cloning techniques described in this chapter.
6. There are several new and improved high-fidelity DNA polymerase enzymes that can be used as alternatives to the enzyme-mentioned in this protocol.
7. Ethidium bromide-DNA complex excitation by UV light may cause photo bleaching of the dye and single-strand breaks. To minimize both effects, use a long-wavelength UV illumination (302 nm instead of 254 nm) to cut the desired DNA bands from the agarose gel.
8. Instead of transformation of *E. coli* by "heat shock," they can be transformed by electroporation following the manufacturer's instructions. However, the presence of salt increases the conductivity of the transformation solution and could cause arcing during the electrical pulse, drastically reducing the transformation efficiency. If arcing occurs, use a smaller amount of the ligation reaction in the electroporation or remove salt from the DNA using a commercial kit or by extraction with phenol:chloroform followed by precipitation with ethanol and 2 M ammonium acetate.
9. Cultures of transformed bacteria should be grown from a single colony isolated from a freshly streaked selective plate. Subculturing directly from glycerol stocks or plates that have been stored for a long time may lead to loss of the construct.
10. Alternatively, aliquot 0.85 mL of bacterial culture medium into a freezing vial and add 0.15 mL of sterile glycerol (sterilized by autoclaving for 20 min at 15 lb./sq. inch on liquid cycle). Vortex the culture to disperse glycerol evenly.

11. Alternatively, scrape the frozen surface of the culture with a sterile plastic pipet tip and then immediately drop the tip into 2 mL of LB broth containing appropriate antibiotics (e.g., ampicillin). Incubate the plate at 37 °C overnight in a shaker incubator.
12. For efficient cell transformation, bacterial culture OD at 550 nm should not exceed 0.8. To ensure that the culture does not grow to a higher density, OD measurement every 20 min after 3 h of growth is highly recommended.
13. Cells must be subconfluent at the time of harvest. Do not overtreat cells with trypsin and do not pipet aggressively. Once cells are on ice, keep them at 4 °C during all subsequent steps and do not let cells settle in the cuvette. After the transcripts and cells are added, proceed to the electroporation within a minute or so.
14. Nucleofactor™ devices (Lonza Walkersville Inc., Walkersville, MD, USA) can provide higher electroporation efficiency compared to standard electroporation units.
15. Most electroporation machines contain programs with defined parameters for transforming specific cell types. In this case, choose the program containing the conditions closest to those described in this protocol.
16. Silent mutations introduced in the viral genome to generate new restriction sites can be used as genetic markers to identify the recombinant virus recovered from the infectious clone.

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Chapter 3

Reverse Genetics for Porcine Reproductive and Respiratory Syndrome Virus

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is a reemerging swine disease, and has become economically the most significant disease in pork production worldwide. The causative agent is PRRS virus (PRRSV), which is a member virus of the family *Arteriviridae*. The PRRSV genome is a single-stranded positive-sense RNA and is infectious. Two strategies in the PRRSV reverse genetics system have been employed for reconstitution of progeny virus: RNA transfection and DNA transfection. The PRRSV reverse genetics has broadly been used for studies including protein structure-function relationship, foreign gene expression, vaccine development, virulence determinants, and viral pathogenesis. Herein, we describe the modification of the pFL12 "RNA launch" reverse genetic system to the CMV promoter-driven pXJ41-FL13 "DNA launch" system. The generation of progeny PRRSV using pXJ41-FL13 is further elucidated.

Key words Porcine reproductive and respiratory syndrome virus, PRRS, Infectious clones, Reverse genetics, Arterivirus, Nidovirus

1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerged and reemerging swine disease, which was firstly reported in the United States in 1987 and subsequently in Europe in 1990. PRRS has quickly spread to most pig-producing countries worldwide and since its emergence has caused significant economic losses to the pork industry [1, 2]. The etiological agent is PRRS virus (PRRSV) and is placed in the family *Arteriviridae* in the order *Nidovirales* together with lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). PRRS viruses isolated from Europe and North America are similar but strikingly differ in their genomic sequences with the sequence similarity of only 55–70%. Hence they form two distinct genotypes: genotype I for European PRRSV and genotype II for North American PRRSV [3, 4].

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The PRRSV genome is a single-stranded positive-sense RNA of 15 Kb in length with 5′ cap and 3′-polyadenylated [poly(A)] tail, which is enclosed in the capsid structure. Two large open reading frames (ORFs), ORF1a and ORF1b, occupy the 5′ three-quarters of the genome and code for two large polyproteins, pp1a and pp1ab. Pp1a is normally translated from ORF1a by ribosomal scanning of the 5′ UTR, but pp1b is translated by the mechanism of −1 frame-shifting in the ORF1a/ORF1b overlapping region to produce the pp1b fusion protein [5]. The pp1a and pp1ab proteins are further processed to generate 14 nonstructural proteins (nsps) by viral proteinases. Recently, a novel ORF has been identified within the nsp2 gene, and this ORF is translated via a −2 ribosomal frame-shift mechanism to produce nsp2TF [6].

The 3′-proximal portion of the genome is compact and organized to contain eight genes, most of which overlap with neighboring genes. These genes code for structural proteins that are translated from the 3′ co-terminal nested set of subgenomic (sg) mRNAs, which is the hallmark of the PRRSV gene expression. Each sg mRNA contains a common leader sequence at their 5′ end that is identical to the 5′-proximal part of the genome and this sequence is referred to as transcription-regulatory sequence (TRS). TRS has a critical role in yielding sg-length minus-strand templates for sg mRNA synthesis via discontinuous transcription, which is a common strategy of Nidovirales [7]. The sg mRNAs are structurally polycistronic but most of them are functionally monocistronic. Notable exceptions are sgmRNA2 and sgmRNA5; they are functionally bicistronic from which E and GP2, and ORF5a and GP5, are expressed, respectively.

The genome from positive-strand RNA viruses is fully infectious, and the reverse genetics system has been developed for many RNA viruses [8]. Two strategies have been developed to generate virus progeny from the full-length cDNA copy of viral genome: RNA transfection and DNA transfection (Fig. 1). In the “RNA launch” approach, in vitro-transcribed viral genome is transfected into cells for the initiation of an infection cycle. In the DNA transfection, the cDNA clone carrying the full-length viral genome is placed under a eukaryotic promoter such as a cytomegalovirus (CMV) promoter, and then introduced to cells.

The first PRRSV infectious clone pABV437 was developed for the genotype I PRRSV Lelystad virus [9]. Numerous infectious clones have since been developed including for the genotype II PRRSV VR-2332 virus, the European-like PRRSV SD01-08 circulating in the United States, and the highly pathogenic PRRSV emerged in China in 2006. PRRSV infectious clones developed early are based on the “RNA launch” strategy. The “DNA launch” strategy has been firstly applied to the P129 infectious clone and this strategy eliminates the need for in vitro transcription [10]. To date, at least 14 different infectious clones are available for PRRSV [8].

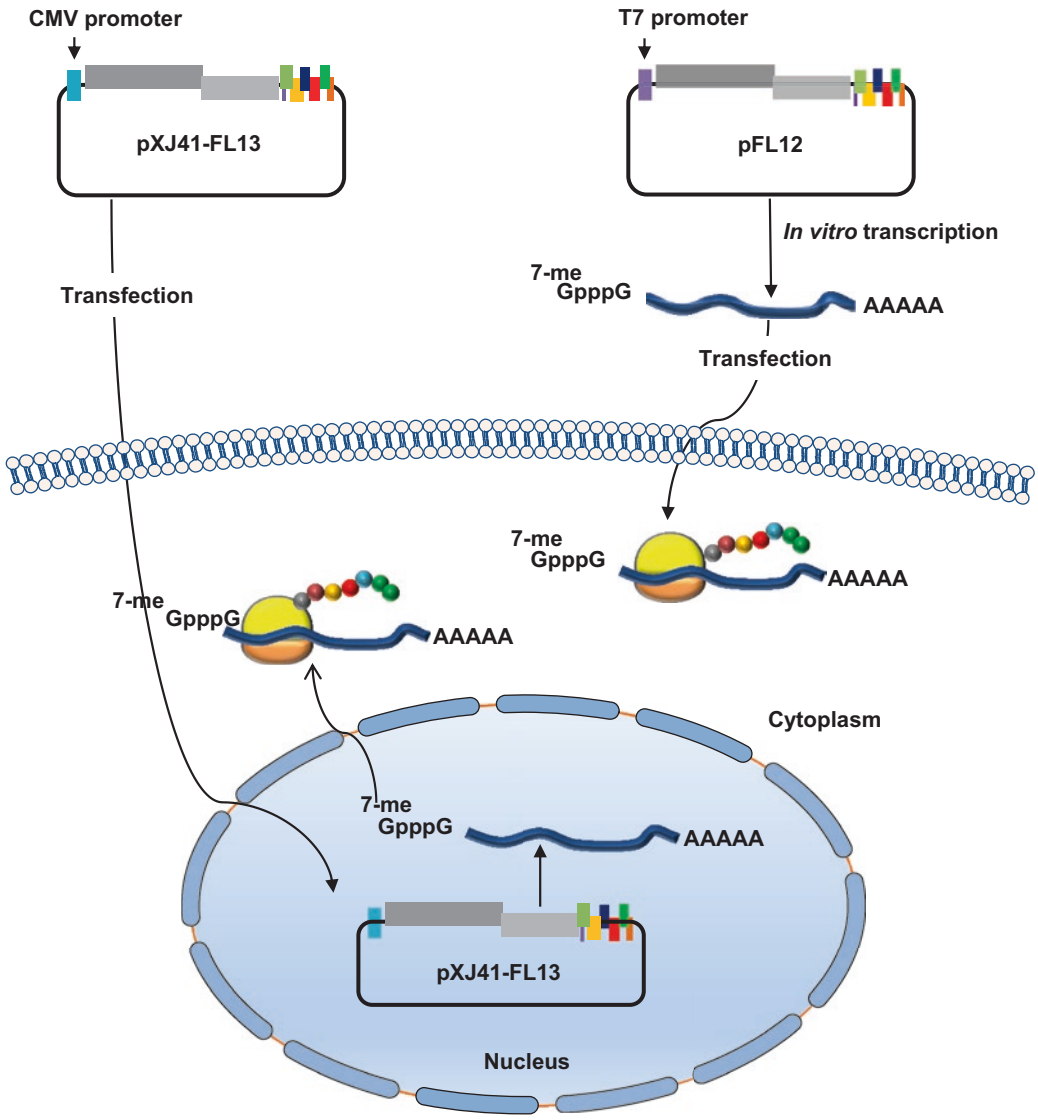

Fig. 1 Production of PRRSV progeny viruses from infectious clones by the "RNA launch" and "DNA launch" strategies. Two strategies have been used for progeny PRRSV generation. In the "RNA launch" strategy, the full-length genomic cDNA is placed under the T7 or SP6 promoter to produce full-length genomic RNA in vitro. The transcribed full-length viral RNA is then transfected to cells to initiate an infection cycle. In the DNA launch strategy, a eukaryotic promoter, typically human cytomegalovirus (CMV) promoter, is placed immediately upstream of the viral sequence, and the full-length cDNA clone is transfected without RNA transcription. Viral genomic RNA is transcribed in the nucleus of transfected cells, which is exported to the cytoplasm to initiate an infection cycle

PRRSV infectious clones have extensively been utilized for the study of biology and vaccinology. The availability of such a powerful molecular tool has facilitated the studies for viral protein functions, virulence and pathogenesis, immune responses, and vaccine development. However, PRRSV genome is unusually complex and

has evolved to optimal fitness. Most of the genetic information seems to be essential, and minor alternations in conserved regions or functional domains appear to be lethal for PRRSV replication. Despite such difficulties, some genetic manipulations for PRRSV have been successful. Mutations, deletions, insertions, and substitutions are major approaches to viral genome manipulation. Modification-tolerant genomic regions have been identified, most of which reside in coding regions for nsp2 and N protein, along with noncoding regions in the genome. Such screening studies have enabled PRRSV to serve as a foreign gene expression vector, and genes such as GFP have been successfully inserted into the PRRSV genome [11]. An additional attempt was made to produce an extra species mRNA for foreign gene expression. A foreign gene fused with a copy of TRS was inserted in the small space between ORF1b and ORF2 [10].

The swapping of structural genes between arteriviruses has been extremely useful to identify viral determinants for cell tropism. The replacement of minor envelope proteins including the E protein with the corresponding genes from EAV allowed the chimeric PRRSV to acquire a broader cell tropism but to lose the ability to infect porcine alveolar macrophages (PAMs). This study indicates that the GP2/GP3/GP4 minor glycoproteins are the viral determinants for cell entry and tropism [12]. In addition, chimeric viruses have been constructed to identify viral determinants for virulence. Swapping nsp9- and nsp10-coding regions between the highly pathogenic and low-pathogenic PRRSV strains proved that these genes are essential for increased pathogenicity and fatal virulence for HP-PRRSV [13].

Reverse genetics system of PRRSV provides a platform for rational design of new PRRSV vaccine. A random sequence shuffling has been employed to generate immunologic variants of PRRSV. This approach has been applied to breed sequences from GP3, GP4-M, GP5, and GP5-M, which allows rapid generation of an attenuated virus and variants inducing broad spectrum of cross-neutralizing antibodies [14–16]. Another approach named synthetic attenuated virus engineering (SAVE) has been used for the rapid generation of attenuated PRRSV [17]. To differentiate naturally infected from vaccinated animals (DIVA) serologically, deletion of an immunodominant epitope from the PRRSV genome has been conducted in an attempt to develop a live attenuated DIVA vaccine for PRRSV. Two epitopes in nsp2 and M have been identified to be useful as a potential DIVA vaccine [18].

Infectious clones are important molecular tools to understand structure-function relationship of proteins and genomic sequences in vivo. Specific sequence motifs may be mutated or deleted from the viral genome using an infectious clone and their phenotypes can be examined to determine their function. Motifs in the nsp1,

nsp2, E protein, and N protein have broadly been studied using mutant viruses [8].

Herein, we describe the construction of the CMV-promoter-based PRRSV infectious clone, pXJ41-FL13, using the previously established T7-promoter-based infectious clone pFL13 which is made for the "RNA-launch" strategy [19]. The new construct pXJ41-FL13 is designed for a "DNA-launch" strategy and is a convenient and powerful tool for structure-function studies of PRRSV.

2 Materials

2.1 Construction of a Full-Length Clone Driven by CMV-Promoter

1. Cloning vector: pXJ41 expression plasmid (*see* **Note 1**).
2. Full-length PRRSV genome: The full-length genome of PRRSV strain NVSL 97-7895 was incorporated into PRRSV infectious clone pFL12 (*see* **Note 2**).
3. Equipments:
   (a) PCR thermocycler.
   (b) 37 °C incubator for bacterial plates.
   (c) Shaking incubator.
   (d) Water bath.
4. PCR primers: The primers used for infectious clone construction are synthesized from Thermo Fisher Scientific. The basic "desalt" grade was sufficient for PCR.
5. Restriction enzymes: They are typically purchased from New England BioLabs (NEB). The enzymes come with 10× reaction buffer and BSA.
6. DNA polymerase: *PfuUltra* High-fidelity DNA Polymerase is purchased from Agilent Technologies.
7. DNA purification kit: QIAquick PCR purification kit is used in this study.
8. Gel extraction kit: QIAquick gel extraction kit is used for DNA purification purposes.
9. T4 DNA Ligase: It is purchased from Invitrogen.
10. Ampicillin stock (100 mg/mL, 1000×) solution: Dissolve 1 g of ampicillin sodium salt in 10 mL of sterilized water.
11. Lysogeny broth (LB) medium for bacterial suspension culture: In 1000 mL of distilled water, dissolve the following ingredients, autoclave, cool to 70 °C, and then add 1 mL of the ampicillin stock (100 mg/mL):
    (a) 10 g Tryptone.
    (b) 5 g Yeast extract.
    (c) 10 g NaCl.

12. Lysogeny broth (LB) agar medium: In 1000 mL distilled water, dissolve the following ingredients, autoclave, cool to 70 °C, add 1 mL ampicillin stock, and then aliquot 25 mL into each Petri dish:
    (a) 10 g Tryptone.
    (b) 5 g Yeast extract.
    (c) 10 g NaCl.
    (d) 15 g Agar.
13. Other supplies for cloning work:
    (a) *E. coli* DH5α competent cells (*see* **Note 3**).
    (b) SOC medium.

2.2 Identification of Infectious Full-Length cDNA Clones

1. Plasmid extraction kit: GeneJET plasmid miniprep kit (Thermo Fisher Scientific).
2. MARC-145 cells.
3. Dulbecco's modified Eagle medium (DMEM).
4. Fetal bovine serum (FBS): 10% FBS is applied for MARC-145 cell growth and 2% FBS is used to maintain virus-infected cells.
5. Transfection reagent:
    (a) Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific).
    (b) Opti-MEM (Thermo Fisher Scientific).
6. Sterile disposables for cell culture work:
    (a) 35 mm Dishes.
    (b) 6-Well plates.
    (c) 5 mL Sterile pipettes.
    (d) 10 mL Sterile pipettes.
    (e) 1000 mL Filter unit (0.20 μm).
    (f) 10 mm diameter of circle cover glasses.
7. Antibodies (*see* **Note 4**):
    (a) SDOW17 mouse monoclonal antibody (mAb).
    (b) PRRSV nsp1β rabbit polyclonal antibody (pAb).
    (c) Alexa-488 anti-mouse IgG.
    (d) Alexa-594 anti-rabbit IgG.
8. Mounting medium: Fluoromount-G mounting medium is purchased from Southern Biotech.
9. 4′,6-Diamidino-2-phenylindole (DAPI): It is purchased from Sigma.

3 Methods

3.1 Construction of the CMV-Promoter Driven PRRSV Infectious Clone pXJ-FL13

1. Plan of pXJ-FL13 construction.

The PRRSV infectious clone pFL12, which contains the full-length genome from PRRSV strain NVSL 97–7895 [19], was chosen for "DNA launch" modification. This strain of virus was isolated from pigs with atypical PRRS in the United States and the genomic sequence is available at GenBank (the GenBank accession number is AY545985.1).

The pXJ41-FL13 cDNA clone consists of two large overlapping fragments (fragment A and fragment B) from the viral genome in addition to the backbone sequence of vector, pXJ41-AP, as described below (Fig. 2).

Fragment A, comprising from the nucleotide positions 1–2550 of the PRRSV genome, was amplified by PCR using pFL12 as the template. Restriction enzyme sites *Asc*I and *Pac*I were designed at the 5′- and 3′-termini of the fragment, respectively (Fig. 2a). Also, the *Spe*I site unique for full-length genomic sequence was included in the fragment A.

The vector pXJ41 was modified to include the restriction enzyme sites *Asc*I and *Pac*I (Fig. 2). The TATA box sequence was added to the upstream of *Asc*I. This modified vector was designated pXJ41-AP. The vector pXJ41-AP and fragment A were digested with restriction enzymes *Asc*I and *Pac*I, and subjected to ligation to generate pXJ41-AP-FragmA.

Fragment B includes the genomic sequence from nucleotide positions 2532 to the 3′-terminus and is prepared from pFL12 using restriction enzymes *Spe*I and *Pac*I. The purified fragment B is ligated to *Spe*I–*Pac*I-digested pXJ41-AP-FragmA to generate the pXJ41-FL13 cDNA clone (*see* **Note 5**).

(a) PCR primer design for fragment A:

Primer F1 (CCAGGGCGCGCCACCGTCATGACG TATAGGTGTTG) is composed of the first 17 bases of the PRRSV NVSL 97-7895 genome and the artificial *Asc*I site (GGCGCGCC, underlined).

Primer R1 (GCCTTAATTAACTGGGCGTTGACT AGT) includes the reverse complement sequence representing the positions 2531–2546, the artificial *Pac*I site (TTAATTAA, underlined), and the *Spe*I site (ACTAGT, underlined).

(b) Construction of the intermediate plasmid pXJ41-AP-FragmA:

100 ng of pFL12 is used as a template. The PCR product of fragment A is digested with *Asc*I and *Pac*I, and ligated with *Asc*I-*Pac*I double-digested pXJ41-AP. The ligation mixture is transformed into competent *E. coli*. Colonies are

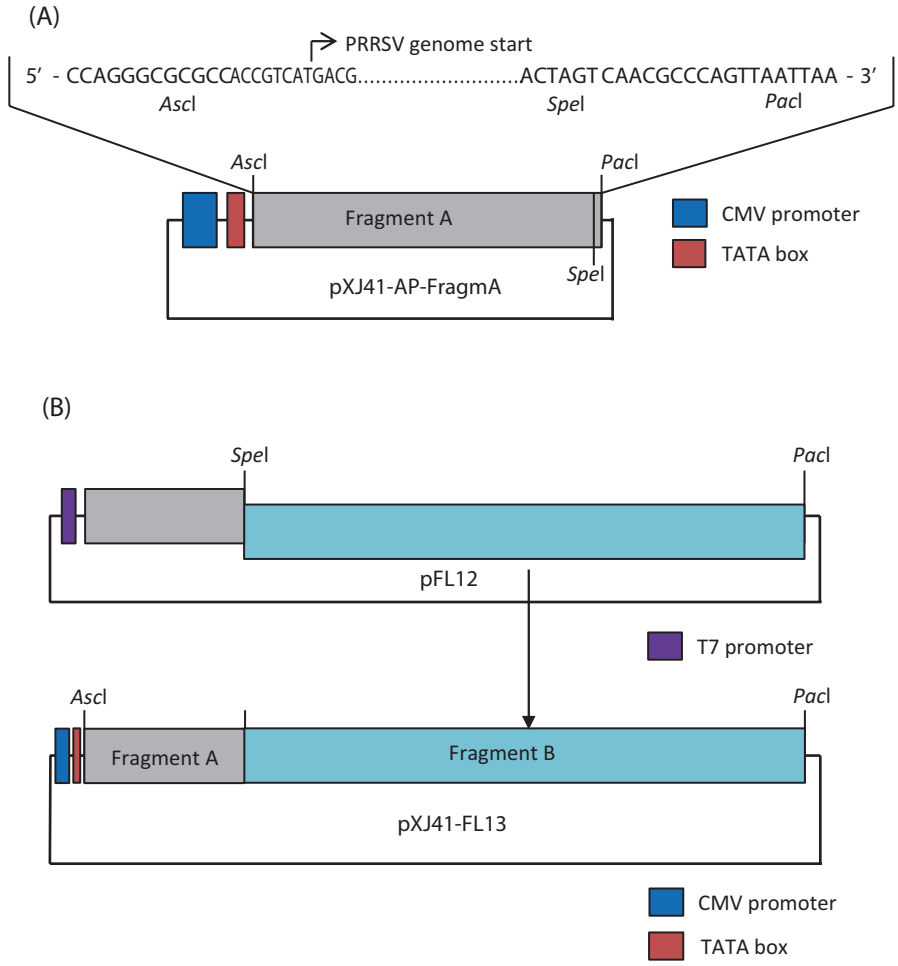

Fig. 2 Construction of the pXJ41-FL13 infectious clone. (**a**) Illustration of the plasmid pXJ41-AP-FragmA. The pXJ41 vector is modified to include two restriction sites, *Asc*I and *Pac*I. Fragment A containing the PRRSV genomic sequence from 1 to 2546 is amplified by PCR and two restriction sequences *Asc*I and *Pac*I are added at the 5′- and 3′-termini, respectively. *Asc*I-*Pac*I double-digested fragment A and pXJ41-AP are ligated together to generate plasmid pXJ41-AP-FragmA. The unique *Spe*I site in the PRRSV genome at position 2532 is included in this plasmid. (**b**) Digest the PRRSV infectious clone pFL12 with *Spe*I and *Pac*I to obtain fragment B. Purified fragment B then is ligated with *Spe*I–*Pac*I-digested pXJ41-AP-FragmA

screened for the presence of the correct insert by restriction analysis and sequencing.

(c) Preparation of fragment B and assembly into a full-length clone:

2 μg of pFL12 is digested with *Spe*I and *Pac*I. The size of fragment B is approximately 13,000 bp and isolated by DNA gel extraction. Fragment B is then ligated with *Spe*I–*Pac*I double-digested and CIP-treated pXJ41-AP-FragmA at a ratio of 3:1, respectively. The ligation mix is transformed to competent *E. coli* DH5α cells. Colonies are

screened for the presence of full-length insert by restriction analysis and sequencing. Ten independent colonies need to be picked and examined for infectivity because of a possible mutation that may have been introduced during PCR.

2. PCR amplification of fragment A.
   (a) Add the following materials into a 0.2 mL PCR tube.

Sterile distilled water	38.0 μL
10× *PfuUltra* High-fidelity reaction buffer	5.0 μL
dNTPs (25 mM each)	1.0 μL
100 ng/μL FL12 cDNA	1.0 μL
10 μM Forward primer	2.0 μL
10 μM Reverse primer	2.0 μL
PfuUltra High-fidelity DNA polymerase (2.5 U/μL)	1.0 μL

   (b) Run PCR under the following parameters: 95 °C for 2 min (95 °C for 30 s, 52 °C for 30 s, 72 °C for 3 min (1 min per 1 kb in length) × 30 cycles, 72 °C for 10-min extension, 4 °C for an indefinite time.

3. Gel purification and restriction digestion of PCR product.
   (a) Prepare a 1% agarose gel in 1× TAE buffer containing ethidium bromide (EthBr).
   (b) Add 5 μL 10× loading dye to 45 μL PCR product and load the mix into a well of the gel.
   (c) Run electrophoresis and visualize DNA bands with a long-wave UV lamp. The target size is approximately 2500 bp.
   (d) Excise the agarose slice containing the desired band with a razor blade and transfer to a 1.5 mL centrifuge tube using a spatula.
   (e) Purify the PCR-amplified DNA fragment from agarose using the QIAquick gel extraction kit according to the manufacturer's instruction.
   (f) Elute the DNA fragment A with 32 μL of DNase-free/RNase-free water.
   (g) Complete digestion of DNA (fragment A) with *Asc*I and *Pac*I restriction enzymes. Add the following materials into a 1.5 mL microcentrifuge tube and incubate at 37 °C for 2 h:

Eluted DNA fragment A	30.0 μL
*Pac*I (15 unit)	1.5 μL
*Asc*I (15 unit)	1.5 μL
NEB CutSmart® Buffer	5.0 μL
Sterile distilled water	12.0 μL

(h) Purify the digested fragment A using the QlAquick PCR purification kit according to the manufacturer's instructions.

4. Restriction digestion and purification of pXJ41-AP.

(a) Complete digestion of DNA fragment A with *Asc*I and *Pac*I restriction enzymes. Add the following materials into a 1.5 mL centrifuge tube and incubate at 37 °C for 2 h (*see* **Note 6**):

pXJ41-AP	1 μg
*Pac*I (15 unit)	1.5 μL
*Asc*I (15 unit)	1.5 μL
NEB CutSmart® Buffer	5.0 μL
Sterile distilled water	to 50 μL

(b) Purify digested pXJ41-AP with the QlAquick PCR purification Kit according to the instructions.

5. Ligation of the fragment A with *Asc*I-*Pac*I-digested pXJ41-AP.

(a) Prepare the following ligation mixture for each PCR DNA fragment:

5× ligase buffer	4.0 μL
Purified PCR fragment A	90 fmol
Purified pXJ4-AP	30 fmol
T4 DNA ligase	1.0 μL
Sterile distilled water	to 20.0 μL

(b) Mix gently and incubate at 16 °C overnight in a PCR machine.

6. Transformation of competent *E. coli* DH5α cells with the ligation mix.

(a) Thaw a tube of competent *E. coli* DH5α on ice.

(b) Chill 1.5 mL centrifuge tubes on ice for 2 min.

(c) Add 50 μL of competent *E. coli* DH5α to the bottom of each tube.

(d) Add 5 μL of ligation mix to the competent cells and swirl the pipette tip gently to mix.

(e) Incubate on ice for 30 min.

(f) Heat-shock the transformation mix at 42 °C for 40 s.

(g) Incubate the transformation tubes on ice for 2 min.

(h) Add 0.95 mL 37 °C SOC to each transformation tube.

(i) Incubate the transformation tubes in a 37 °C shaker for 1 h at 200 rpm.

(j) Plate the transformation mix on LB agar plates containing 100 μg/mL ampicillin.

(k) Incubate plates in a 37 °C incubator overnight.

7. Screen for clones containing the correct insert.

(a) Pick five colonies and inoculate each colony into 5 mL LB/ampicillin broth in a Falcon 15 mL conical centrifuge tube.

(b) Incubate tubes in a 37 °C shaker overnight at 200 rpm.

(c) Extract plasmid DNA using the GeneJET plasmid miniprep kit according to the manufacturer's instructions.

(d) Perform digestion with *Asc*I and *Pac*I restriction enzymes in a 25 μL reaction as described above.

(e) Prepare a 1% agarose gel in 1× TAE buffer containing ethidium bromide.

(f) Add 2.5 μL 10× loading buffer to 22.5 μL of reaction and then load the mixture on the gel.

(g) Run electrophoresis and then visualize DNA bands with a long-wave UV lamp. The target size is approximately 2500 bp.

(h) Keep the plasmid with the correct insert and send for sequencing.

8. Preparation of *Spe*I–*Pac*I-digested pXJ41-AP and fragment B.

(a) Double-digest pXJ41-AP and pFL12 with *Pac*I and *Spe*I. Add the following materials into a 1.5 mL centrifuge tube and incubate at 37 °C for 2 h:

pXJ41-AP or pFL12	1 μg
*Pac*I (15 unit)	1.5 μL
*Spe*I (15 unit)	1.5 μL
NEB CutSmart® Buffer	5.0 μL
Sterile distilled water	To 50 μL

(b) Purify the fragment B by agarose gel electrophoresis using the QIAquick gel extraction kit according to the manufacturer's instruction. The fragment B is 12,932 bp in length.

(c) Purify *Spe*I–*Pac*I double-digested pXJ41-AP with the QIAquick PCR purification kit according to the instructions.

9. Construction of full-length cDNA clone.

(a) Ligate *Spe*I–*Pac*I double-digested pXJ41-AP and fragment B at a ratio of 1:3, as described in **Step 6** of Subheading 3.1.

(b) Transform the ligation mixture into competent *E. coli* DH5α cells, as described in **Step 7** of Subheading 3.1.

(c) Pick ten colonies and inoculate each colony into 5 mL LB/ampicillin broth in a Falcon 15 mL conical centrifuge tube.

(d) Incubate tubes in a 37 °C shaker overnight at 200 rpm.

(e) Extract plasmid DNA using the GeneJET plasmid miniprep kit according to the instructions. Dissolve plasmid DNA of each isolate in 50 μL DNase/RNase-free water and measure the concentration by NanoDrop.

(f) Screen colonies for the presence of the full-length insert by restriction analysis using *Spe*I and *Pac*I, as described in **Step 8** of Subheading 3.1 (*see* **Note 7**).

(g) Keep the correct plasmid at −20 °C for later use.

3.2 Rescue of Progeny PRRSV from an Infectious Clone

1. Transfection of plasmid into MARC-145 cells (*see* **Note 8**).

(a) Prepare 80–90% confluent MARC-145 cell monolayers in 35 mm dishes.

(b) Prepare transfection mixtures at RT:

- Add 125 μL Opti-MEM into the bottom of a 1.5 mL centrifuge tube.
- Add 4 μL Lipofectamine 2000 directly into Opti-MEM, and tap the tube gently to mix.
- Incubate the Lipofectamine/Opti-MEM mixture at room temperature (RT) for 5 min.
- Add 125 μL Opti-MEM into the bottom of another 1.5 mL centrifuge tube.
- Add 2 μg of plasmid DNA into Opti-MEM, and then tap the tube gently to mix.
- Combine the plasmid and Lipofectamine 2000, and then tap the tube gently to mix well.
- Incubate the transfection mixture at RT for about 20 min.

(c) Prepare MARC-145 cell monolayer for transfection (while incubating the transfection mix):

- Remove the culture medium.
- Wash cell monolayer with 1 mL Opti-MEM, and then completely aspirate off the medium.
- Add 1 mL Opti-MEM to monolayer.

(d) Add 0.25 mL of the transfection mixture onto the monolayer.

(e) Swirl dish gently 2–3 times to disperse the transfection mixture.

(f) Incubate the dishes at 37 °C for 6 h.

(g) Replace transfection medium with 2 mL of DMEM containing 2% FBS.

(h) Continue to incubate the dishes at 37 °C for 5 days.

2. Preparation of passage 1 progeny virus in MARC-145 cells.

(a) Freeze the culture dishes at −80 °C for 10 min to lyse the cells and then thaw the frozen dishes.

(b) Repeat the freeze-thaw one more time and scrape the cell monolayer at RT using a cell scraper.

(c) Remove cell debris by spinning in a refrigerated microcentrifuge at 4 °C for 10 min at 13,400 × *g*.

(d) Transfer the clarified supernatant into cryogenic vials and then store at −80 °C until use for infectivity assay. This stock is designated as passage 1 (P1) PRRSV.

3. Identification of virus infectivity and preparation of passage 2 virus (*see* **Note 9**).

(a) Prepare MARC-145 cells in a 6-well plate and grow to 90% confluency.

(b) Remove media from each well of MARC-145 cells by aspiration.

(c) Inoculate 200 μL of P1 virus per well.

(d) Incubate the plate at 37 °C for 1 h to allow virus infection.

(e) Add 800 μL DMEM containing 2% FBS.

(f) Incubate the plate at 37 °C.

(g) Check daily and record appearance of cytopathic effect (CPE) (Fig. 3a). CPE may gradually appear on day 2 or 3.

(h) Collect culture supernatant at day 5, and designate P2 virus. Repeat **Steps 1**–**8** in the same way to prepare passage 3 virus (P3). Store the virus at −80 °C for further characterization.

4. Identification of infectivity by immunofluorescence assay (*see* **Note 10**).

(a) Place one or two round coverslips per well in a 12-well plate. Prepare MARC-145 cells in the plate and grow cells to 70% confluency.

(b) Remove the culture medium by aspiration.

(c) Inoculate cells with 200 μL of P1 or P2 virus per well.

(d) Incubate plates at 37 °C for 1 h to allow infection.

(e) Add 800 μL DMEM containing 2% FBS.

(f) Incubate the plates at 37 °C for 2 days.

(g) Remove the culture medium and wash monolayers with PBS three times. Fix cells with 4% paraformaldehyde for 1 h.

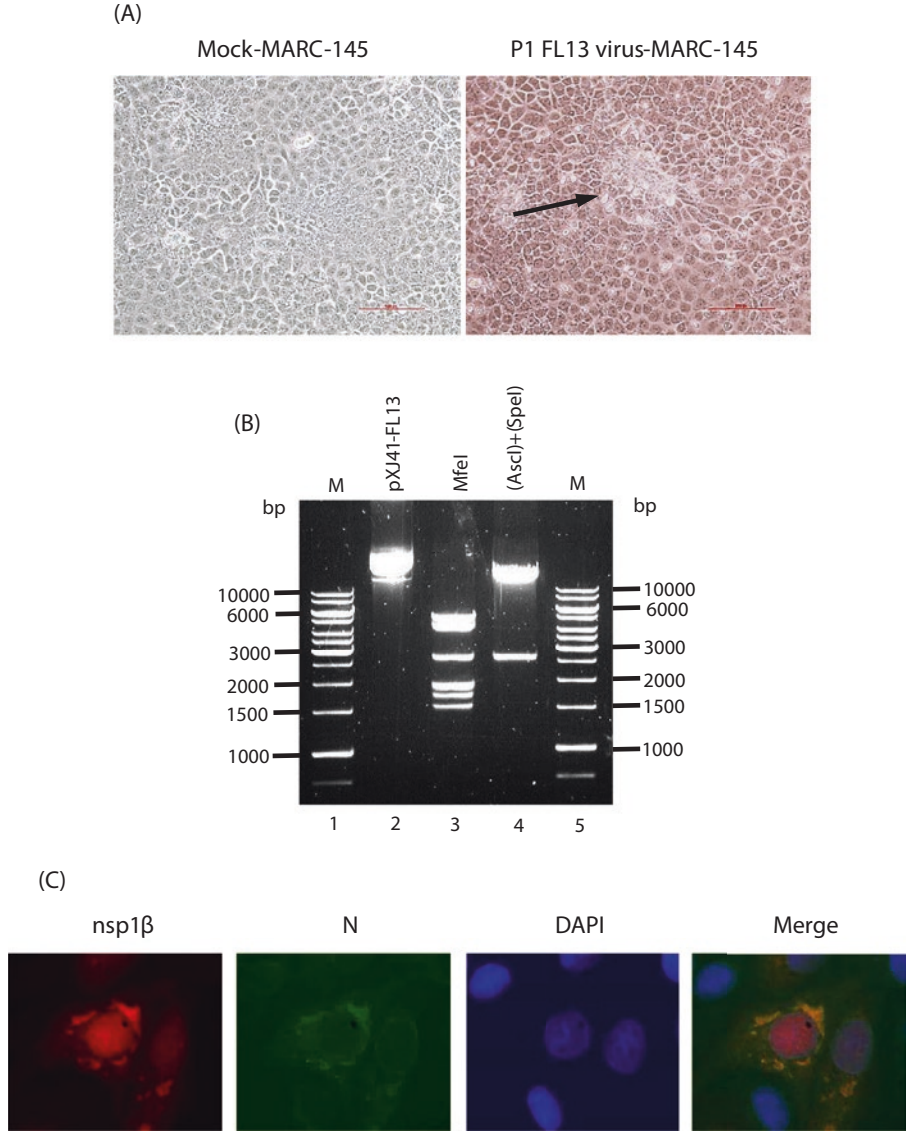

Fig. 3 Infectivity of PRRSV rescued from the pXJ41-FL13 infectious clone. (**a**) Appearance of CPE in MARC-145 cells on 2 days post-infection with P1 virus. P1 virus obtained from transfection with pXJ-FL13 was added to MARC-145 cells at 90% confluency in a 6-well plate. Images are taken 2 days post-infection. *Black arrowhead* indicates CPEs. (**b**) Restriction patterns of the pXJ-41-FL13 infectious clone on 1% agarose gel. Lanes 1 and 5 (M), molecular weight markers in bp. Lane 2 (FL13), undigested pXJ-41-FL13 infectious clone. Lane 3, pXJ-41-FL13 digested with *Mfe*I. Lane 4, pXJ-41-FL13 double-digested with *Asc*I and *Spe*I. The digestion was carried out using 2 μg of plasmid and 1 unit of respective enzymes in 30 μL at 37 °C for 4 h. (**c**) Expression of PRRSV proteins in MARC-145 cells transfected with pXJ-41-FL13. At 4 days post-transfection with pXJ-41-FL13, cells were fixed and stained with SDOW17 mAb specific for N and polyclonal antibody (pAb) specific for nsp1β

(h) Permeabilize cells with 0.1% Triton X-100 for 15 min.

(i) Wash cell monolayers with PBS three times.

(j) Block cells by incubating with 1% BSA in PBS for 30 min at RT.

(k) Incubate the coverslips with anti-N mouse mAb and anti-nsp1β rabbit pAb for 1 h.

(l) Incubate with Alexa Fluor 488- and/or Alexa Fluor 568-conjugated secondary antibody for 1 h.

(m) Stain the nucleus by incubating coverslips with DAPI for 3 min at RT.

(n) After washing with PBS, coverslips are mounted on microscope slides using Fluoromount-G mounting medium.

(o) Examine the staining signal using a fluorescent microscope.

4 Notes

1. As a backbone plasmid vector for infectious clone construction, a low-copy-number plasmid is generally preferred as suggested in some studies [9, 20], but it appears unnecessary. The pXJ41 vector is a high-copy plasmid and used for eukaryotic expression. The vector pXJ41 is ampicillin resistant.
2. The infectious clone pFL12 is 19,213 nucleotides in length and contains the full-length genomic sequence of PRRSV strain NVSL 97-7895 which was placed under the T7 promoter [19]. The genome of NVSL 97-7895 shares 90–91% sequence identity with North American strains VR-2332, PA-8, and 16244B. A unique restriction enzyme site *BsrGI* was introduced in the genome to serve as the genetic marker.
3. Competent *E. coli* DH5α is used to amplify the intermediate construct pXJ41-AP-FragmA and the infectious clone pXJ41-FL13. Transformation of DH5α produces colonies harboring pXJ41-FL13, but bacteria grow slowly, which is probably due to the large size of plasmid. It is not recommended to store transformed bacterial stock at −80 °C since we have noticed that some viral sequence is lost during the storage. The infectious clone may be prepared from fresh bacterial culture after transformation with the full-length cDNA plasmid.
4. SDOW17 is anti-PRRSV N mAb specific for the nucleocapsid (N) protein, and the dilution of 1:200 is used for immunofluorescence staining. Anti-PRRSV-nsp1β rabbit polyclonal antibody (pAb) is specific for nsp1β of North American genotype PRRSV. It was generated at the Immunological Research Centre, University of Illinois at Urbana-Champaign. A dilution of 1:300 is used for immunofluorescence.

5. Since the pFL12 infectious clone is available in the scientific community, it is desirable to replace the backbone plasmid with pXJ41 to fulfill the use of CMV promoter to drive PRRSV genome transcription. No convenient restriction enzyme site is present upstream of pFL12. *Spe*I and *Pac*I, which are located at positions 2531–2536 and downstream of the poly(A) tail of PRRSV genome, are chosen to obtain the largest fragment of the viral genome by restriction cleavages. This approach reduces the risk of introducing potential mutations by PCR.
6. To reduce background during screening, overnight incubation is suggested to achieve complete restriction digestion of the vector.
7. In addition to double digestion using *Spe*I-*Asc*I, *Mfe*I digestion is an alternative enzyme to screen colonies for the correct insert. *Mfe*I digestion has been used for pFL12 screening and the expected sizes of digestion fragments are 1525, 1704, 1882, 1906, 2617, 4527, and 5034 bp (Fig. 3b) [19].
8. BHK-21, MA-104, and MARC-145 are the cells of choice for transfection and progeny virus production. Although BHK-21 cells are nonpermissive for PRRSV infection, they provide a high efficiency of transfection and a good production of progeny. Supernatants collected from BHK-21 cells may be used to infect MARC-145 cells for amplification.
9. CPE may not be obvious after transfection with the infectious clone even at 5 days of incubation. To determine clear CPE, supernatant (P1 virus) collected from transfected cells may be used to infect fresh MARC-145 cells for 5 days. CPE will appear.
10. In transfected cells, viral protein production is negligible and it is difficult to predict the generation of infectious progeny. Fresh MARC-145 cells infected with P1 virus will give a positive signal after staining with anti-PRRSV antibodies, which will provide a reliable indication of infectivity of progeny derived from infectious clones. The detection of nsp1β and N proteins by staining indicates definite production of nonstructural proteins and structural proteins, respectively (Fig. 3c).

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Chapter 4

Reverse Genetics of Zika Virus

Chao Shan, Xuping Xie, and Pei-Yong Shi

Abstract

We have established a reverse genetic system for Zika virus (ZIKV). Five shuttle plasmids were constructed and assembled into the full-length cDNA clone of ZIKV genome. To ensure the stability of the cDNA clone, we used a low copy vector (pACYC177) and a set of unique restriction enzyme sites on the ZIKV genome to assemble the full-length cDNA clone. A T7 promoter was engineered in front of the viral 5′ UTR for in vitro transcription. A hepatitis delta virus ribozyme (HDVr) sequence was engineered following the viral 3′ UTR for generation of the authentic 3′ end of the RNA transcript.

Key words Flavivirus, Zika, Mosquito, Dengue, West Nile, Guillain-Barré syndrome, Microcephaly

1 Introduction

The current explosive epidemic of Zika virus (ZIKV) in the Americas poses a global public health emergency. ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family, which includes significant human pathogens such as yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and dengue virus (DENV). ZIKV is closely related to the four serotypes of DENV with approximately 43% amino acid identity [1].

The ZIKV genome comprises a single-strand, positive-sense RNA genome of about 11,000 nucleotides which encodes three structural proteins (capsid [C], pre-membrane/membrane [prM/M], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form viral particles. The nonstructural proteins participate in viral replication, virion assembly, and evasion of host immune response [2]. ZIKV is mainly transmitted by *Aedes spp.* mosquitoes, which also transmit YFV and DENV, as well as chikungunya virus (an emerging alphavirus). In addition, ZIKV may also be transmitted through sex, blood transfusion, organ transplantation,

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and potentially through urine or saliva [3, 4]. Individuals with compromised immunity could be more susceptible to ZIKV infection and disease development [5].

The first isolation of ZIKV is from the blood of a sentinel rhesus monkey in the Zika forest of Uganda in 1947 [6]. ZIKV has predominantly been associated with sylvatic transmission cycles between primates and arboreal mosquitoes in forests, and has for six decades (1947–2006) rarely caused human diseases, with only 13 naturally acquired cases reported [7]. It has been estimated that up to 80% of ZIKV infections are asymptomatic. Patients with symptomatic ZIKV infection usually present with a mild febrile illness characterized by fever, lethargy, conjunctivitis, rash, and arthralgia. However, recent outbreaks undergoing in the South Pacific and the Americas have caused severe diseases, including Guillain-Barré syndrome and congenital microcephaly [8]. Genomic sequencing has indicated two lineages ZIKV: the ancestral African lineage and Asian lineage. The Asian lineage is responsible for the recent/current epidemics: it caused an epidemic on Yap Island, Micronesia, in 2007; it then spread from an unknown source, probably in Southeast Asia, to French Polynesia and other regions of the South Pacific and caused large epidemics in 2013–2014; subsequently, ZIKV arrived in the Americas in 2015 and led to millions of human infections. The reasons for the rapid spread of ZIKV are unknown. Experimental systems, including a reverse genetic system of ZIKV, animal models, and mosquito transmission models, are urgently needed to address these key scientific questions.

For construction of the cDNA clone, ZIKV RNA from Cambodian strain FSS13025 was sequenced (GenBank number KU955593.1) and used as the template to construct the infectious cDNA clone. Five RT-PCR fragments (A–E) spanning the complete viral genome were individually cloned and assembled into the full-length cDNA of ZIKV, named as pFLZIKV (Fig. 1). Based on our previous experience with infectious clones of other flaviviruses [9–11], we chose a low-copy-number plasmid pACYC177 (15 copies per *E.coli* cell) to clone fragments A and B as well as to assemble the full-genome cDNA. This plasmid was used because fragments A and B, spanning the viral prM-E-NS1 genes, were toxic to *E. coli* during the cloning procedure; high-copy-number vectors containing these fragments were unstable, leading to aberrant deletions/mutations of the inserts [9]. In contrast, fragments C, D, and E were not toxic to *E. coli*, and could be cloned individually into a high-copy-number plasmid pCR2.1-TOPO. A T7 promoter and a hepatitis delta virus ribozyme (HDVr) sequence were engineered at the 5′ and 3′ ends of the complete viral cDNA for in vitro transcription and for generation of the authentic 3′ end of the RNA transcript, respectively.

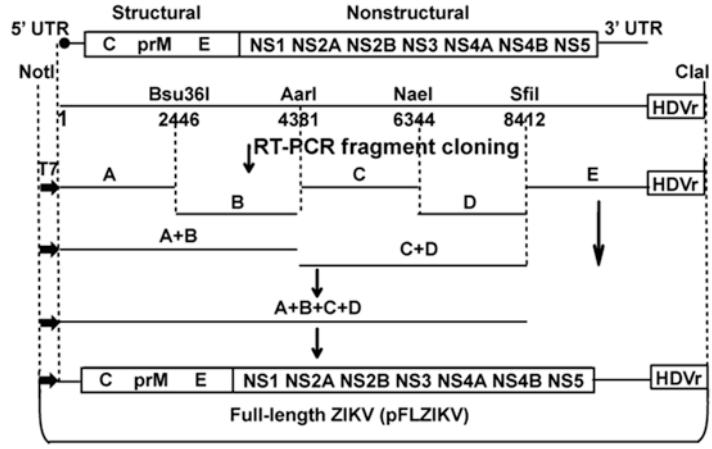

Fig. 1 The strategy for constructing the full-length cDNA clone of ZIKV. Five fragments from A to E were used to assemble the full-length cDNA of ZIKV

2 Materials

2.1 Propagation of Large Stock of ZIKV on Vero Cells

1. Vero cells.
2. PBS (Gbico).
3. 1× Dulbecco's modified Eagle medium (DMEM) high glucose with 4 mM L-glutamine (Thermo Fisher Scientific).
4. Fetal Bovine Serum (HyClone).
5. Penicillin-Streptomycin (Gibco).
6. Trypsin-EDTA (0.25%), phenol red (Gibco).
7. Serological pipettes (Corning).
8. T175 flask with vent cap (Corning).
9. Filter tips (Thermo Fisher Scientific).

2.2 Plaque Assay of ZIKV

1. Carboxymethylcellulose sodium salt (Sigma-Aldrich).
2. Dulbecco's modified Eagle medium high glucose, powder (Thermo Fisher Scientific).
3. DMSO (Sigma-Aldrich).
4. Fetal Bovine Serum (HyClone).
5. Penicillin-Streptomycin (Gibco).
6. HEPES (Thermo Fisher Scientific).
7. Crystal violet (Sigma-Aldrich).
8. Formaldehyde (Sigma-Aldrich).
9. 10 mL Combitips (Eppendorf).

2.3 Extraction of Viral RNA from Virus

1. QIAamp Viral RNA Mini Kit (Qiagen).
2. Ethanol absolute (Sigma-Aldrich).

2.4 Amplification the Fragments Spanning Zika Virus Genome by RT-PCR

1. SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity DNA Polymerase (Thermo Fisher Scientific).
2. Nuclease-free water (Ambion).

2.5 Subcloning and Assembly of the Full-Length Zika cDNA Clone

1. Restriction endonuclease (New England Biolabs).
2. T4 DNA ligase (New England Biolabs).
3. Top10 competent cells (Thermo Fisher Scientific).
4. LB agar containing 100 μg/mL ampicillin.
5. LB media containing 100 μg/mL ampicillin.
6. 37 °C Incubator (Thermo Fisher Scientific).
7. Shaker.
8. QIAGEN Plasmid Plus Maxi Kit (Qiagen).

2.6 Linearization of the ZIKV cDNA Clone

1. Phenol-chloroform solution (Thermo Fisher Scientific).
2. Chloroform (Sigma-Aldrich).
3. 3 M Sodium acetate (pH 5.2).
4. 70% Ethanol.
5. 100% ethanol (Sigma-Aldrich)
6. Tabletop refrigerator centrifuge (Eppendorf).

2.7 In Vitro Transcription of ZIKV RNA

1. mMESSAGE mMACHINE T7 Transcription Kit (Ambion).
2. Nuclease-free water (Ambion).
3. Microcentrifuge tubes 1.7 mL (Axygen).
4. 37 °C Incubator.
5. Nuclease-free tips.

2.8 Transfection into the Vero Cells and Rescue ZIKV

1. Ingenio electroporation solution (Mirus).
2. 4 mm Cuvette.
3. Hemocytometer.
4. GenePulser apparatus (Bio-Rad).
5. Nunc® Lab-Tek® Chamber Slide™ system (Sigma-Aldrich).
6. Vero cells.
7. In vitro-transcribed ZIKV RNA.
8. PBS (Gbico).
9. 1× Dulbecco's modified Eagle medium (DMEM) high glucose with 4 mM L-glutamine (Thermo Fisher Scientific).

10. Fetal Bovine Serum (HyClone).
11. Penicillin-Streptomycin (Gibco).
12. Trypsin-EDTA (0.25%), phenol red (Gibco).
13. Serological pipettes (Corning).
14. T175 flask with vent cap (Corning).
15. Filter tips (Thermo Fisher Scientific).

3 Methods

3.1 Infection of the Vero Cells with ZIKV

1. Thaw the virus stock on ice.
2. Infect T175 flask of 90% confluent monolayers of Vero cells with 100 μL ZIKV stock.
3. Incubate at 37 °C with 5% CO_2 for 2 h to allow the virus to infect the cells.
4. After 2 h of incubation, replace the media with DMEM with 2% FBS containing 1% penicillin-streptomycin.
5. On day 3 post-infection, harvest all the supernatant and transfer them into 50 mL Falcon tube.
6. Spin the supernatant with 500 × *g* 5 min to remove any cell debris and store the aliquot at −80 °C.

3.2 Plaque Assay of ZIKV

1. Place 150 μL of undiluted samples into wells of columns 1 and 7, and put 135 μL of diluent into remaining wells (Fig. 2).

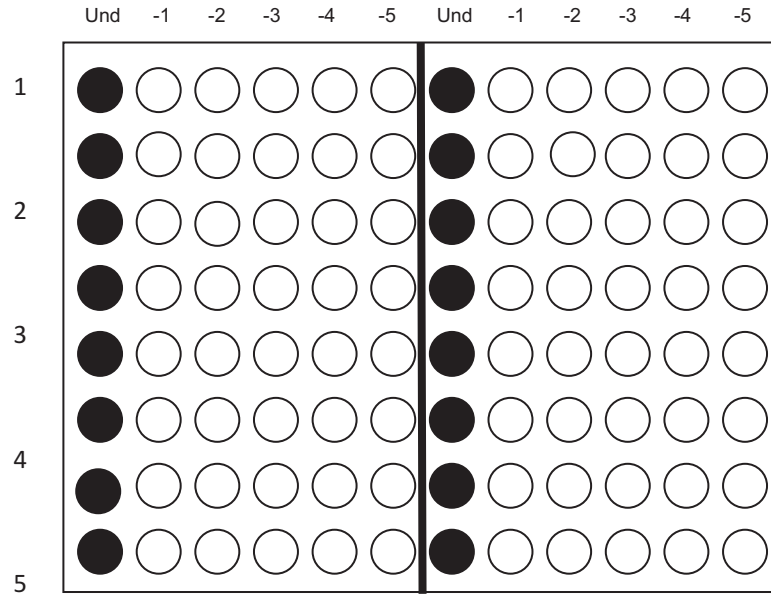

Fig. 2 Diagram of dilutions for ZIKV plaque assay

Alternatively, put 135 μL of diluent into all wells and then put 15 μL of undiluted virus samples into wells of columns 1 and 7, making −1 dilution. Mix the samples thoroughly and be consistent among dilutions.

2. Using a multichannel pipette, transfer 15 μL from column 1 to column 2. Change tips, mix the samples in column 2 by pipetting up and down several times, and transfer 15 μL from column 2 to column 3.
3. Continue with these steps through column 6, changing tips between each transfer. Repeat the procedure for columns 7–12. If necessary, adjust protocol to dilute virus further.
4. Label 24-well plates. Dump the media into a waste container (*see* **Note 1**).
5. Starting with the lowest dilution, transfer 100 μL of diluted virus to the corresponding well on a 24-well plate. The same tip can be used for all dilutions of the same virus.
6. Gently rock the plate to evenly distribute the inoculum and incubate for 1 h at 37 °C and 5% CO_2.
7. Using repeater, add 0.5 mL of methyl cellulose to each well (pipette the methylcellulose on the side of well. DO NOT DIRECTLY PIPETTE ON THE CELLS) (*see* **Note 2**). Incubate the plates at 37 °C and 5% CO_2 for 4 days.
8. Soak the plates into 3.7% formaldehyde, and fix for 30 min.
9. Wash the plates with tap water. Make sure to wash out all of the methylcellulose.
10. Stain the plate with 1% crystal violet for 5 min.
11. Wash the plates by tap water and count the plaques.

3.3 Extraction of the Viral RNA from ZIKV

1. Pipette 560 μL of buffer AVL into a 1.5 mL microcentrifuge tube and add 5.6 μL carrier RNA.
2. Add 140 μL cell-culture supernatant to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
3. Incubate at room temperature (15–25 °C) for 10 min. Viral particle lysis is complete after lysis for 10 min at room temperature.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 μL of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s.

6. Carefully apply 630 μL of the solution from **step 5** to the QIAamp Mini column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 × *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 mL collection tube, and discard the tube containing the filtrate. Close each spin column to avoid cross-contamination during centrifugation.
7. Carefully open the QIAamp Mini column, and repeat **step 6**.
8. Carefully open the QIAamp Mini column, and add 500 μL of Buffer AW1. Close the cap, and centrifuge at 6000 × *g* (8000 rpm) for 1 min.
9. Carefully open the QIAamp Mini column, and add 500 μL of Buffer AW2. Close the cap and centrifuge at full speed (20,000 × *g*; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini column in a new 2 mL collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 μL of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 × *g* (8000 rpm) for 1 min. Viral RNA is stable for up to 1 year when stored at −80 °C.

3.4 Amplification of the Fragments by RT-PCR

1. Use the primer pairs to amplify corresponding regions.
2. Add the following to a 0.2 mL, nuclease-free, thin-walled PCR tube on ice. For multiple reactions, you can prepare a master mix to minimize reagent loss and enable accurate pipetting.

Reagents	Volume (μL)
Nuclease-free water	17
2× Reaction mix	25
Primer F	1
Primer R	1
RNA	5
SuperScript® III RT/Platinum® Taq Mix*	1
Total volume	50

3. Gently mix, making sure that all the components are at the bottom of the amplification tube. Centrifuge briefly if needed.

4. Place the reaction in the thermal cycler programmed as described below.

Reaction conditions	
55 °C	30 min
94 °C	2 min
94 °C 60 °C 68 °C	15 s 30 s } 40 cycles 2 min 30 s (1 Kb/min)
68 °C	5 min
4 °C	Forever

5. Run the 0.8% gel to check the RT-PCR products.
6. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
7. Weigh the gel slice in a colorless tube. Add three volumes of Buffer QG to one volume of gel (100 mg–100 μL) .
8. Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.
9. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose) (*see* **Note 3**).
10. Add one gel volume of isopropanol to the sample and mix.
    Do not centrifuge the sample at this stage.
11. Place a QIAquick spin column in a provided 2 mL collection tube.
12. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
13. Discard flow-through and place QIAquick column back in the same collection tube.
14. Collection tubes are reused to reduce plastic waste.
15. (Optional) Add 0.5 mL of Buffer QG to QIAquick column and centrifuge for 1 min.
16. To wash, add 0.75 mL of Buffer PE to QIAquick column and centrifuge for 1 min.
17. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at >10,000 × *g* (~13,000 rpm).
18. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.

19. To elute DNA, add 50 μL of Buffer EB (10 mM Tris·Cl, pH 8.5) or MQ water to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed (*see* **Note 4**).

3.5 Subcloning and Assembly of the Full-Length cDNA Clone

1. Double digest the RT-PCR products from Subheading 3.4 with corresponding restriction endonuclease.
2. Ligate the corresponding fragments with related backbones.
3. Transform into the Top10 competent cells.
4. Verify the inserts by DNA sequencing.
5. Assemble the whole genome following Fig. 2.

3.6 Linearization of the Full-Length cDNA Clone

1. Linearization for the cDNA of ZIKV

   100 μL reaction system (per sample) was usually recommended.

ddH_2O	Up to 100 μL
10× NEB buffer CutSmart	10 μL
Enzyme	100 U (ClaI, according to the enzyme activity unit)
DNA plasmid	10 μg

2. Incubate at 37 °C water bath for 2 h. Analyze by agar gel electrophoresis with 1 μL loaded.
3. Phenol-chloroform extraction of ClaI-linearized ZIKV cDNA plasmid.
4. Add equal volume of phenol:chloroform 100 μL to the reaction system.
5. Vortex for 5 s and centrifuge for 1 min at 13,000 rpm.

   Note: After centrifuge, there are two layers in the liquid and gently take out the tube from centrifuge.
6. Take off the top layer carefully and transfer to a new tube.
7. Back extraction: Add 100 μL ddH_2O into the previous tube including phenol:chloroform; vortex for 5 s and centrifuge for 1 min at 13,000 rpm.
8. Take off the top layer (~100 μL) carefully and transfer to the tube from step 6 including 100 μL DNA. Therefore, the total of liquid volume is 200 μL.
9. Add equal volume of chloroform (200 μL), vortex for 5 s, and centrifuge at 13,000 rpm for 10 min.
10. Take off the top layer and transfer to another new tube.
11. Add 1/10 volume of 3 M NaAc (20 μL) , add 3× volume of cold 100% ethanol (−20 °C pre-cold), and gently mix well.
12. Precipitate at −20 °C for ≥20 min or overnight.

13. Spin for 15 min at 4 °C. Add 1 mL 70% ethanol to wash the pellet gently. Centrifuge for 15 min at 4 °C.
14. Air-dry. Add nuclease-free water (15 μL) to dissolve the linearized plasmids.
15. Load 1.5 μL DNA solutions to NanoDrop and quantify the linearized plasmid concentration.

3.7 In Vitro Transcription to Generate RNA

1. Thaw the frozen reagents.
2. Place the RNA Polymerase Enzyme Mix on ice; it is stored in glycerol and will not be frozen at −20 °C.
3. Vortex the 10× reaction buffer, 2× NTP solution, and GTP solution.
4. All reagents should be spun briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.
5. Assemble transcription reaction at room temperature following the table below (*see* **Note 5**):

Component	Amount
Nuclease-free water	To 20 μL
2× NTP solution	10 μL
GTP solution	1 μL
10× Reaction buffer	2 μL
Linear template DNA	0.1–1 μg
Enzyme mix	2 μL

6. Mix thoroughly.
7. Gently flick the tube or pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.
8. Incubate at 37 °C, for 2 h.
9. After 2-h incubation, add 1 μL TURBO DNase, mix well, and incubate for 15 min at 37 °C.
10. Stop the reaction and precipitate the RNA by adding 30 μL nuclease-free water and 30 μL LiCl precipitation solution.
11. Mix thoroughly. Chill for ≥30 min at −20 °C or overnight.
12. Spin for 15 min at 4 °C. Add 1 mL 70% ethanol to wash the pellet gently. Centrifuge for 15 min at 4 °C. The white pellet should be seen.
13. Add nuclease-free water (100 μL) to dissolve the RNA (*see* **Note 6**).
14. Load 1.5 μL RNA solutions to NanoDrop and quantify the RNA concentration.

3.8 Transfection into the Vero Cells and Rescue of the Virus

1. Warm up PBS, trypsin, and medium.
2. Prechill a 4 mm cuvette and a bottle of PBS, and keep them on ice.
3. Thaw 10 μg of viral RNA on ice.
4. Remove supernatant from monolayer Vero cells and wash with warm PBS once.
5. Add 4 mL pre-warmed trypsin to the washed monolayer, incubate at room temperature for 1 min, and tap flask to detach cells.
6. Add 10 mL DMEM with 10% FBS and 1% P/S to the flask, and pipette the cells up and down to disperse cells.
7. Transfer the cells to a 50 mL Falcon tube, and centrifuge at 415 × *g* (1380 rpm) for 5 min at 4 °C.
8. After centrifugation, remove supernatant, and disperse cells by tapping the tube with finger gently (*see* **Note 7**).
9. Resuspend cells in cold PBS, count cells using hemacytometer, keep only 10 cells and centrifuge at 415 × *g* (1380 rpm) for 5 min at 4 °C.
10. Adjust Vero cells to 10^7 cells/mL in Mirus buffer.
11. Add 10 μg RNA to the cuvette.
12. Transfer 8 × 10^6 cells (800 μL) to a prechilled 4 mm cuvette, and keep cuvette on ice.
13. Mix gently.
14. Perform electroporation using the Gene Pulser (0.45 kV, 25 μF), three pulses with 3-s intervals.
15. Leave the electroporated cells at room temperature for 10 min.
16. Resuspend the electroporated cells in the 24 mL DMEM with 10% FBS and 1% P/S. Seed the cells into the 8-well chambers to check the viral protein expression. Transfer the rest of the content to a T-75 flask and incubate at 37 °C with 5% CO_2.

3.9 Plaque Assay to Examine the Recombinant Virus from Cell Culture

Follow Subheading 3.2.

4 Notes

1. Do not remove media from more than one 24-well plate at once in case the cells were dried.
2. To prevent the overlay detaching the cells, do not add it directly on middle of the well. Instead add the overlay on the side of the wall.
3. If the color of the mixture is orange or violet, add 10 μL of 3 M sodium acetate, pH 5.0, and mix. The color of the mix-

ture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH <7.5. Buffer QG contains a pH indicator which is yellow at pH <7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

4. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at −20 °C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris–Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
5. The spermidine in the 10× reaction buffer can coprecipitate the template DNA if the reaction is assembled on ice. Add the 10× reaction buffer after the water and the ribonucleotides are already in the tube.
6. Do not let the pellet dry as it is hard to dissolve the RNA when it is dried.
7. Do not use pipette to disperse the cells as they are fragile now.

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Chapter 5

Efficient Reverse Genetic Systems for Rapid Genetic Manipulation of Emergent and Preemergent Infectious Coronaviruses

Adam S. Cockrell, Anne Beall, Boyd Yount, and Ralph Baric

Abstract

Emergent and preemergent coronaviruses (CoVs) pose a global threat that requires immediate intervention. Rapid intervention necessitates the capacity to generate, grow, and genetically manipulate infectious CoVs in order to rapidly evaluate pathogenic mechanisms, host and tissue permissibility, and candidate antiviral therapeutic efficacy. CoVs encode the largest viral RNA genomes at about 28–32,000 nucleotides in length, and thereby complicate efficient engineering of the genome. Deconstructing the genome into manageable fragments affords the plasticity necessary to rapidly introduce targeted genetic changes in parallel and assort mutated fragments while maximizing genome stability over time. In this protocol we describe a well-developed reverse genetic platform strategy for CoVs that is comprised of partitioning the viral genome into 5–7 independent DNA fragments (depending on the CoV genome), each subcloned into a plasmid for increased stability and ease of genetic manipulation and amplification. Coronavirus genomes are conveniently partitioned by introducing type IIS or IIG restriction enzyme recognition sites that confer directional cloning. Since each restriction site leaves a unique overhang between adjoining fragments, reconstruction of the full-length genome can be achieved through a standard DNA ligation comprised of equal molar ratios of each fragment. Using this method, recombinant CoVs can be rapidly generated and used to investigate host range, gene function, pathogenesis, and candidate therapeutics for emerging and preemergent CoVs both in vitro and in vivo.

Key words Coronavirus (CoV), Reverse genetics, Severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), Emerging, Preemergent, Bat coronavirus, Porcine epidemic diarrhea virus (PEDV)

1 Introduction

Human coronaviruses (HCoVs) were first identified in the 1960s (HCoV-229E and HCoV-OC43) and were primarily associated with mild upper respiratory tract infections with the potential to progress to a severe respiratory disease in young children, the elderly, and immunocompromised individuals [1]. Although additional HCoVs were known to circulate at this time, these strains were not culturable; therefore, HCoV-229E and HCoV-OC43

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infections modeled our understanding of CoV disease severity until 2003 [1]. In Southeast Asia, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in late 2002 and caused acute respiratory distress syndrome and an age-dependent mortality rate of 10–50%, clearly demonstrating that HCoVs were emerging pathogens with pandemic potential [2]. The portent of a worldwide pandemic mobilized the scientific community, leading to robust public health intervention strategies that controlled the epidemic. Moreover, the outbreak spurred academic interest into CoV gene function and pathogenic mechanisms associated with SARS-CoV-induced disease, leading to the development of therapeutic countermeasures. Because of the availability of reverse genetics, robust in vitro replication, and in vivo animal models of human disease, SARS-CoV has become the most intensively studied prototype for HCoV research [3]. SARS-CoV research prompted the generation of novel animal models that have provided insight into (1) genetic changes in the SARS-CoV genome that modulate respiratory pathogenesis [4]; (2) the impact of SARS-CoV on host innate and adaptive immune responses [5, 6]; (3) the role of host genes in regulating SARS-CoV pathogenesis in mice [4, 7]; and (4) novel strategies for the development of vaccines and therapeutic countermeasures [3].

Two novel HCoVs (NL63 and HKU1) were identified shortly after the emergence of SARS-CoV [8–10], and nearly a decade later, in 2012, the world saw the emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) (Fig. 1). MERS-CoV causes acute respiratory distress syndrome (ARDS), severe pneumonia-like symptoms, and multi-organ failure, with a case fatality rate of ~36% [11]. Human cases of MERS-CoV have been predominantly observed in Saudi Arabia and the Middle East. MERS-CoV-infected individuals have also traveled internationally, illustrating the potential for global spread. For example, a South Korean native returning home from the Middle East in May 2015 initiated an outbreak that infected 186 people, resulting in 20% mortality and a nationwide economic crisis [12]. Transmission of MERS-CoV has been mostly observed among health care workers in the hospital setting. Accumulating evidence indicates that Middle Eastern individuals working in close contact with dromedary camels are at increased risk of acquiring MERS-CoV [13]. Camels are suspected to be intermediate hosts between bats and humans that can repeatedly allow for reemergence of MERS-CoV in the human population. Though camels show only mild symptomology during MERS-CoV infection, zoonotic CoV infections can be highly pathogenic in animals. Demonstrated recently by the emergence of a porcine CoV in the United States, porcine epidemic diarrhea virus (PEDV) has caused severe disruption to the pork industry with the deaths of tens of millions of animals in the first 2 years and a >90% mortality rate [14] (Fig. 1). PEDV,

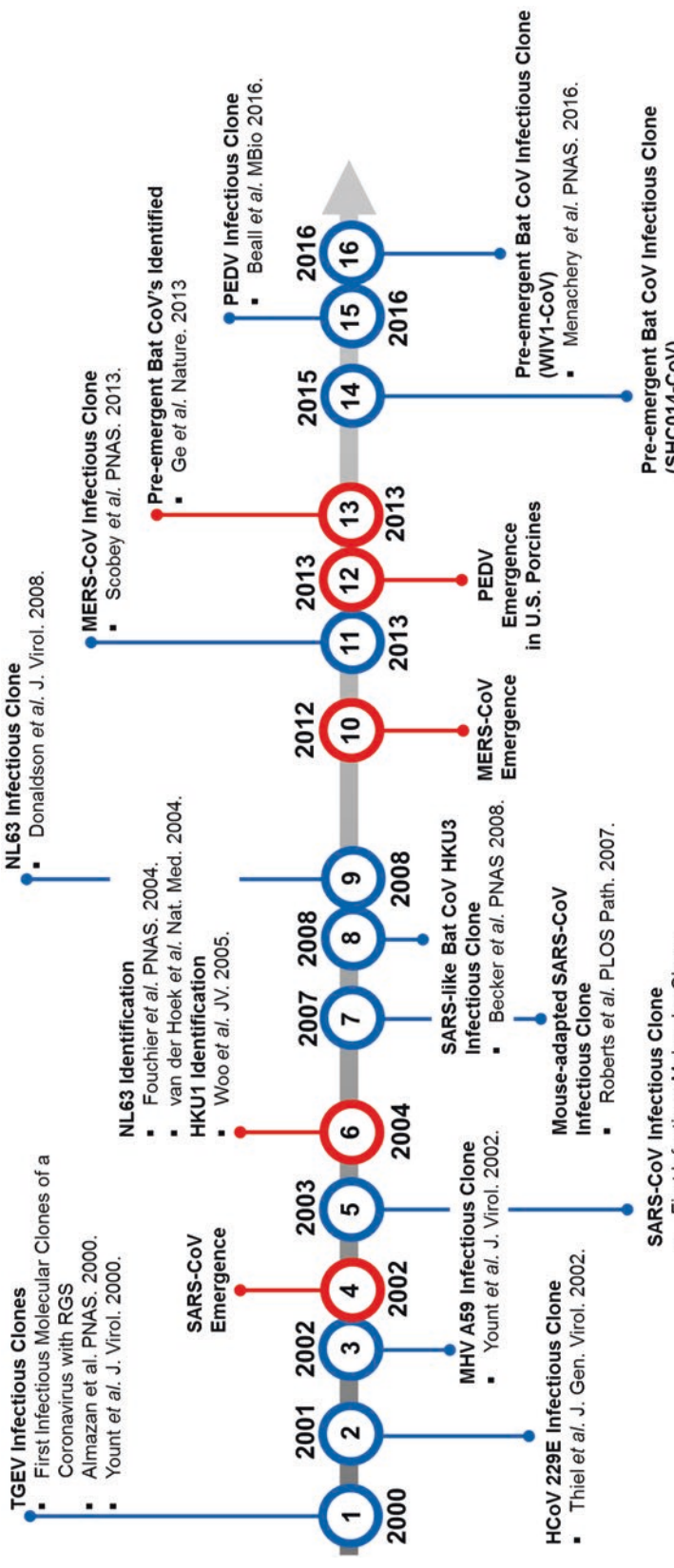

Fig. 1 Timeline of emerging coronavirus events and infectious clones generated using reverse genetics systems (RGS). RGS timeline spans from the first RGS clones, TGEV, in 2000 through the most recently generated RGS clone, preemergent bat coronavirus infectious clone (WIV1-CoV), in 2016. *Red* indicates outbreak and viral identification events. *Blue* indicates the publication of RGS clones. Transmissible gastroenteritis virus (TGEV), mouse hepatitis virus (MHV), severe acute respiratory syndrome coronavirus (SARS-CoV), Hong Kong University (HKU), Middle East respiratory syndrome coronavirus (MERS-CoV), porcine epidemic diarrhea virus (PEDV)

MERS-CoV, SARS-CoV, HCoV NL63, HCoV HKU1, and 229E-HCoV are thought to have emerged from a bat reservoir, while OC43-HCoV is thought to have originated among bovine coronaviruses. In 2013 preemergent SARS-like CoVs were identified in horseshoe bats and found to be poised for entry into the human population [15] (Fig. 1). Importantly, much of the HCoV research over the last 15 years has been possible because of the capacity to generate infectious clones using highly efficient reverse genetics platforms [16], coupled with robust small animal models of human disease [17, 18].

Reverse genetic systems for coronaviruses were difficult to achieve because of the large size of the viral RNA genome, genome stability in bacterial vectors, difficulty in driving full-length 30 kb RNA transcripts in vitro, poor transfection efficiencies, and low infectivity of the viral genome. In 2000, the first molecular clone was developed for transmissible gastroenteritis virus (TGEV), using targeted splice junctions to increase genome stability in low-copy baculovirus vectors [19] (Fig. 1). A few months later, our group published an alternative TGEV reverse genetic strategy [20], and then again applied this technique for the group 2 murine coronavirus [21]. A final innovation in CoV molecular clone design was the insertion of full-length HCoV 229E molecular clone into vaccinia virus in 2001 [22]. Each approach has proven to be a potentially powerful system to probe the role of CoV genes in replication and pathogenesis [3, 23, 24]. This review primarily focuses on the reverse genetic strategy developed in the Baric laboratory, a technology that partitions the CoV genome into discrete fragments, and uses class IIS and IIG restriction endonucleases to systematically and seamlessly assemble full-length cDNA genomes of CoVs. After in vitro transcription and transfection of full-length genomes into permissive cells, recombinant viruses are recovered which contain the genetic content of the molecular clone.

Although the reverse genetic system (RGS) described here achieved prominence shortly after the emergence of SARS-CoV [16], this platform has been used to generate CoV infectious clones that span nearly the entire breadth of the *Coronaviridae* family, including pathogenic viruses from groups 1a and 1b of the *alphacoronaviruses* and groups 2a, 2b, and 2c of the *betacoronaviruses* (Fig. 1). The entire CoV fragments are joined by type IIS or IIG restriction sites (e.g., BglI, SapI, and BsaI) that support directional, seamless ligation into full-length genome (Fig. 2). For type IIS (e.g., SapI) restriction enzymes, the recognition sequences are separated from its cleavage site by one or more variable nucleotides, leaving three to four nucleotide unique overhangs (Fig. 2). Thus, these enzymes leave 64–256 unique ends, providing directionality during multi-segment assembly. Moreover, the recognition site is not palindromic, allowing for seamless assembly of component cDNA clones into full-length genes and genomes. By orienting the

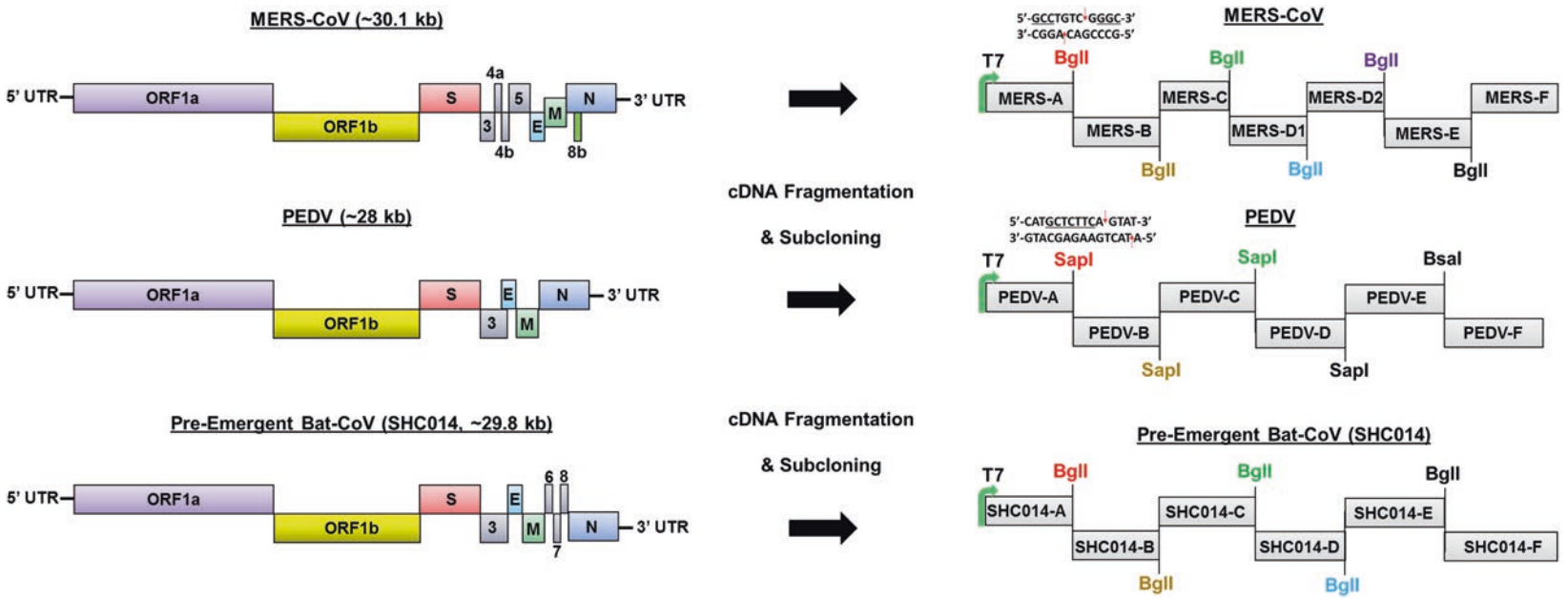

Fig. 2 Organization of coronavirus genomes and infections clones used to generate recombinant coronavirus. (**a**) *Left*: Genome organization of MERS-CoV, PEDV, and SHC014. *Right*: Organization of coronavirus cDNA fragments used in subcloning in order to generate a genome-length cDNA template prior to transcription. Color-coded restriction sites denote distinct type IIS (SapI) or type IIG (BglI) restriction sites. An example of each is shown for the first junction encoded in the MERS-CoV and PEDV genomes. (**b**) A benefit of the RGS infectious clone system is the ease of directed genome mutation. By swapping fragments between wild-type and mutant MERS-CoV, or even between various coronavirus species, useful CoV variants such as wild-type spike or open reading frame mutants can be rapidly generated in order to understand the role of specific mutations or viral genes. Multiple infectious clones with different genetic mutations can be generated in parallel provided that the mutations are on different fragments. *Example*: Three different viruses are generated from mutations in the nsP3 gene (fragment A, *blue*) and the spike gene (fragment E, *purple*)

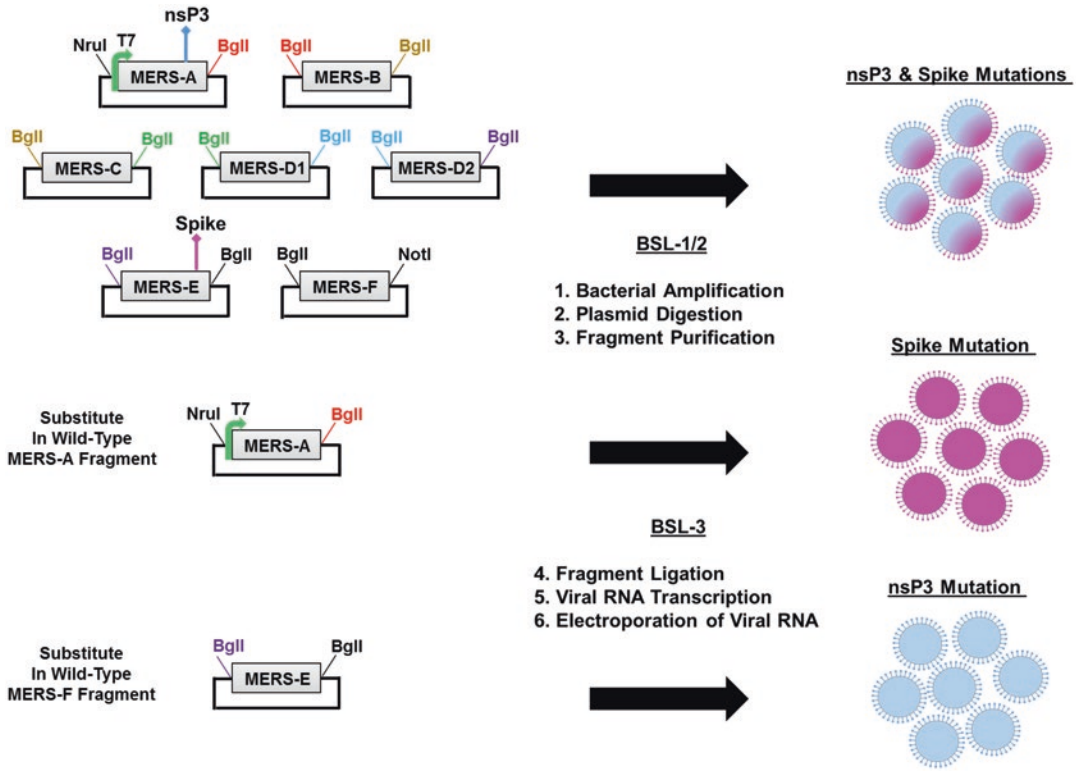

Fig. 2 (continued)

recognition sequence external to the cleavage site, upon digestion and ligation, the restriction site is lost, seamlessly joining the cDNAs, while preserving ORF integrity and sequence authenticity. A second approach uses type IIG (e.g., BglI) restriction endonucleases, which has a palindrome restriction site, bisected by a variable domain of five nucleotides and also leaves 64 different overhangs for directional assembly of large genome molecules (Fig. 2). In this instance, the restriction site is retained in the assembled product. As coronavirus genomes are unstable in bacteria, junctions are oriented within toxic sequence domains, thereby bisecting region toxicity and increasing component clone stability. Plasmids are digested with type IIS or IIG restriction enzymes to isolate each fragment of the CoV genome (Fig. 2). Fragments are then resolved on an agarose gel, purified, and ligated (Fig. 2). Following ligation, the coronavirus genome-length mRNA is in vitro transcribed from a T7 promoter added at the 5′ end of the 5′ UTR. In some instances, strong T7 stop sites are mutated to promote full-length transcript synthesis in in vitro transcription reactions [20]. Resulting genome-length mRNA is electroporated into a permissive mammalian cell line (Fig. 2). Cloning success and viral fitness can be measured by plaque assay and growth curves (Fig. 3). During viral replication

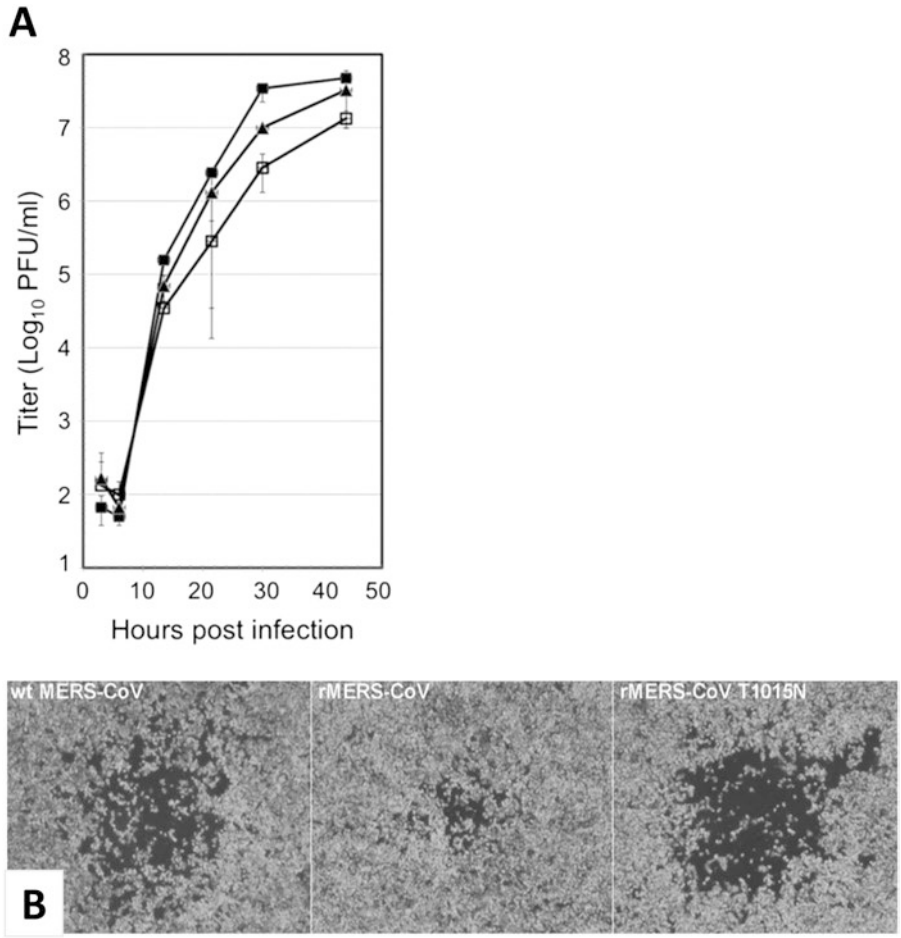

Fig. 3 Confirmation of MERS-CoV production and growth characteristics. (**a**) A comparison of growth curves in wild-type (*filled square*, MERS-CoV), recombinant MERS-CoV (*open square*, rMERS-CoV) and a recombinant MERS-CoV containing a tissue culture-adapted mutation in the spike gene (*filled triangle*, rMERS-CoV-T1015N). (**b**) A comparison of plaque formation in wild-type MERS-CoV, rMERS-CoV, and rMERS-CoV-T1015N. The recombinant MERS-CoV with the tissue culture adaptation cloned back in using RGS exhibits plaque sizes similar to wild-type MERS-CoV. All images reprinted from [25]

CoVs have the unique capacity to generate a nested set of subgenomic viral RNAs (sgRNAs) harboring similar 5′ and 3′ untranslated regions (UTRs) (Fig. 4). Northern blot analysis of CoV RNA, and/or PCR of viral cDNA, affords visualization of sgRNA and confirmation of replication-competent virus (Fig. 4). The efficiency of RGS is exemplified by the fact that an infectious clone of SARS-CoV (icSARS) was generated and published [16] within weeks of the initial publication of the SARS-CoV Urbani strain sequence [26] (Fig. 1). The use of DNA synthesis companies to synthesize a full-length coronavirus genome was achieved in 2008, using HCoV NL63 as a model [27], and then applied to MERS-CoV and various bat SARS-like CoVs in 2008, 2013, 2015, and 2016 [25, 28–30].

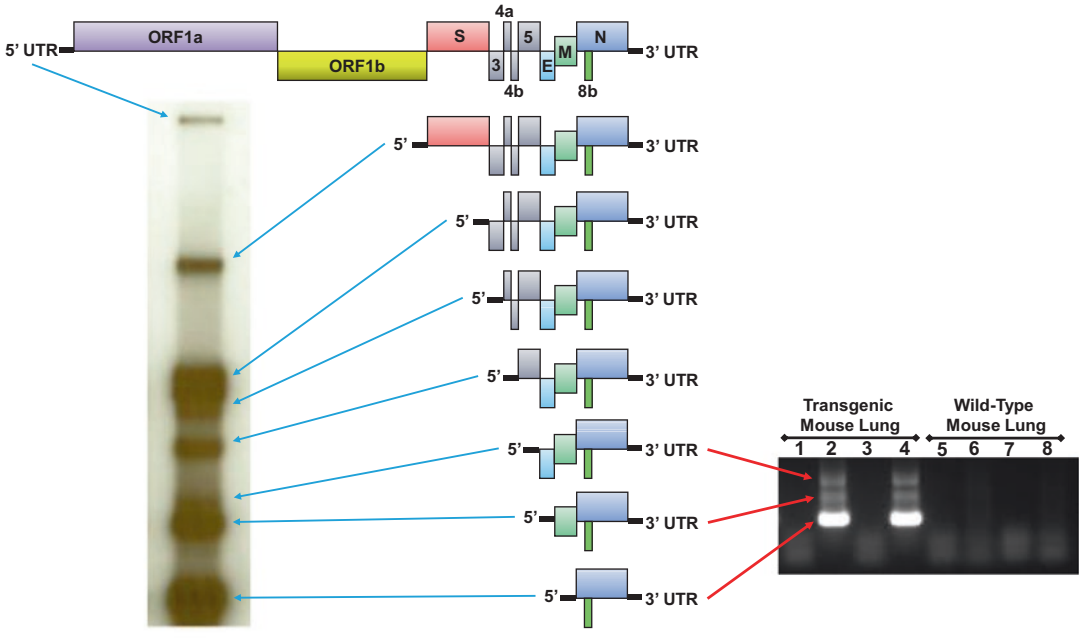

Fig. 4 MERS-CoV subgenomic RNA (sgRNA). sgRNA is generated during active coronavirus replication and can be visualized by northern blot (*left*) and PCR (*right*). Using a biotinylated or radioactive probe against the CoV N-gene, all sgRNA species can be visualized by running RNA isolated from CoV-infected cells on a northern gel (*left*). Northern blot image is reprinted from [25]. Alternately, PCR primers contained within the leader sequence and N-gene can be used to generate PCR products from viral cDNA in order to visualize sgRNA, signifying productive CoV replication (*right*). Here, PCR products confirm productive replication of MERS-CoV in the lungs of a mouse permissive to MERS-CoV infection (transgenic mouse lung), but not in a nonpermissive, wild-type mouse lung. Both of these methods can be used to confirm productive CoV replication following RGS clone generation

Finally, our group has applied this approach to generate stable molecular clones for flaviviruses that include dengue and the newly emerged Zika virus [31, 32].

Importantly, fragmenting HCoV genomes confers additional biological safety benefits over reverse genetics systems that would otherwise maintain a CoV genome as a cDNA molecule comprising greater than 2/3 of the full-length genome. As sanctioned by "NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines), April, 2016" Section III-E-1 indicates that "Recombinant or synthetic nucleic acid molecules containing no more than two-thirds of the genome of any eukaryotic virus may be propagated and maintained in cells in tissue culture using BL1 containment." Moreover, full-length CoV genomes that encode select agents such as SARS-CoV are further regulated under the Federal Select Agent Program (www.selectagents.gov), and must be handled according to specific guidelines.

Partitioning the SARS-CoV genome allows for efficient handling of genomic fragments under standard BSL1/2 containment conditions (Fig. 2). For reconstruction of full-length genomes encoding CoVs restricted to BSL3 containment (SARS-CoV, MERS-CoV, preemergent bat CoVs), fragment ligation and all subsequent steps are executed under BSL3 conditions, including recovery of recombinant viruses (Fig. 2).

1.1 Applications of the Reverse Genetics Platform

One advantage of a segmented molecular clone design is that mutagenesis can occur in parallel on multiple fragments, and that the individual fragments can be "reassorted" to make larger panels of derivative mutants encoding mutation subsets (Fig. 2). For example, an early application included the introduction of over 27 mutations into the SARS-CoV genome at 9 different genome transcription regulatory sequences, thereby demonstrating for the first time that the transcription regulatory circuit of a virus could be rewired [33]. The applicability of the RGS was ratified in ensuing reports describing the cause-and-effect relationship between site-directed mutations introduced into the viral genome which altered viral fitness and in vivo pathogenesis phenotypes [17, 18, 33–36].

In a seminal pathogenesis study RGS was used to validate that six mutations acquired during mouse adaptation of the SARS-CoV Urbani (MA15) strain indeed caused lung pathology associated with severe acute respiratory distress syndrome [18]. Since these mutations were dispersed across the entire genome, RGS proved to be an efficient method to introduce all six mutations simultaneously in order to generate a robust mouse-adapted SARS-CoV strain. Notably, because MA15 SARS-CoV is a novel virus strain, RGS is an effective method to validate Koch's postulates by demonstrating a cause-and-effect relationship. After nearly a decade of research the MA15 strain continues to play a dominant role in SARS-CoV mouse pathogenesis studies, including vaccine and therapeutic evaluations [7, 17, 37–39]. In today's research environment these studies would be considered gain-of-function (GOF) studies, and thereby would have been subject to increased government oversight, which would have inevitably stymied progress into understanding the molecular mechanisms governing emerging coronavirus pathogenesis, host range, receptor usage, virus replication, and vaccine and therapeutic efficacy [3]. Notably, SARS-CoV gain-of-function (GOF) studies have yielded invaluable information regarding the role of viral proteins in pathogenesis in animal models and in tissue culture studies [17, 36]. Applying the combined technologies of GOF studies with the RGS will be essential to future research on emergent and preemergent coronaviruses.

More recently RGS applications were extended to MERS-CoV wherein a replication-competent MERS-CoV expressing the tomato red fluorescent protein (tRFP) was rapidly generated (reproduced from [25]) (Fig. 5). The infectious clone (icMERS-CoV-tRFP) has

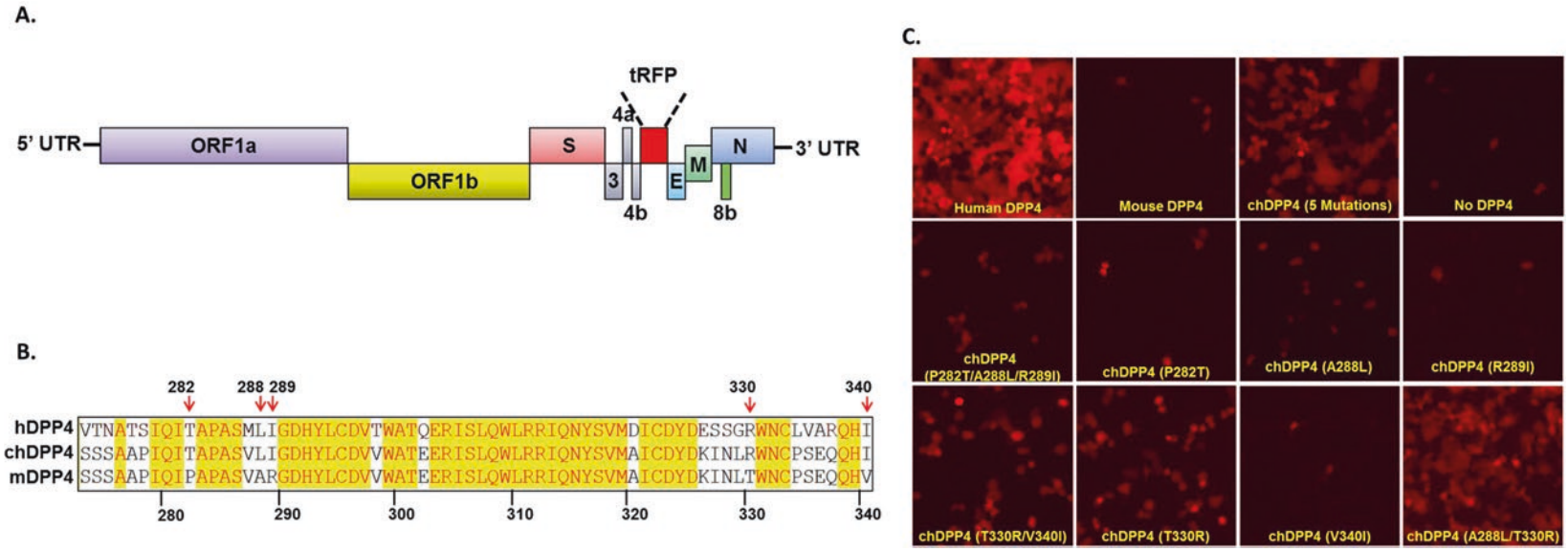

Fig. 5 Application for an infectious clone of MERS-CoV expressing tRFP. (**a**) Genomic representation of an infectious clone of MERS-CoV containing the tomato red fluorescent protein (tRFP) substituted for the orf 5 gene (icMERS-CoV-tRFP). (**b**) Protein sequence alignment of human, mouse, and chimeric dipeptidyl peptidase 4 (DPP4) MERS-CoV host receptor sequence with five specific amino acid changes indicated by *red arrows*. Figure reprinted from [40]. (**c**) The indicated chimeric DPP4 receptor constructs were overexpressed in 293T cells and subsequently infected with the icMERS-CoV-tRFP virus to map the minimal number of amino acids required to facilitate MERS-CoV infection. Two amino acid changes (A288L and T330R) were sufficient to support infection and replication of MERS-CoV. Figure reprinted from [40]

since been applied to understanding host factors that restrict MERS-CoV infection [40, 41]. Figure 5 demonstrates how the icMERS-CoV-tRFP was used to facilitate mapping of specific amino acids in the host dipeptidyl peptidase 4 (DPP4) receptor that are necessary to permit MERS-CoV infection and replication [40]. Humanizing the mouse DPP4 by changing a minimum of two amino acids (A288L and T330R) on the mouse DPP4 protein conferred efficient infection/replication of MERS-CoV as determined by expression of tRFP from the icMERS-CoV-tRFP virus (Fig. 5) [40]. The following protocol outlines the RGS used to generate infectious clones of MERS-CoV, and provides detailed methods for building recombinant coronaviruses using this technique.

2 Materials

2.1 Transformation and Restriction Screening of the Seven MERS-CoV Fragments

1. Seven plasmids containing MERS fragments A–F cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA) or pUC57 vectors.
2. Appropriate restriction enzymes and buffers for screening each MERS fragment (*see* Table 1).
3. Chemically competent bacterial cells (Top 10, Invitrogen).
4. LB broth supplemented with carbenicillin (100 μg/mL) or kanamycin (50 μg/mL) for growth of bacterial cells following plasmid transformation.
5. LB plates supplemented with carbenicillin (100 μg/mL) or kanamycin (50 μg/mL) for growth of bacterial cells following plasmid transformation.
6. Qiaprep Spin Miniprep Kit (Qiagen).

Table 1
Fragmenting the MERS-CoV genome over seven plasmids

Fragment	Plasmid vector	Antibiotic	Flanking restriction sites 5′–3′	Restriction digest buffer	Insert length (bp)
MERS-A	pUC57	Carbenicillin	NruI[a], Bgl I	NEBuffer 3.1	4692
MERS-B	pUC57	Carbenicillin	Bgl I, Bgl I	NEBuffer 3.1	4118
MERS-C	pUC57	Carbenicillin	Bgl I, Bgl I	NEBuffer 3.1	3446
MERS-D1	pCR-XL-TOPO	Kanamycin	Bgl I, Bgl I	NEBuffer 3.1	3211
MERS-D2	pCR-XL-TOPO	Kanamycin	Bgl I, Bgl I	NEBuffer 3.1	3335
MERS-E	pUC57	Carbenicillin	Bgl I, Bgl I	NEBuffer 3.1	5590
MERS-F	pUC57	Carbenicillin	Bgl I, Not I[a]	NEBuffer 3.1	5721

Each genomic fragment is indicated with its corresponding plasmid backbone, the antibiotic used for plasmid selection, the restriction enzymes and buffer used for cloning each fragment, and the length of each MERS-CoV-encoded fragment
[a]Available as high-fidelity enzymes from New England BioLabs, and digested in CutSmart Buffer

7. Gel electrophoresis-grade agarose.
8. TAE buffer: 40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA.
9. Ethidium bromide, or other DNA-visualizing dye.
10. DNA ladder that allows for determination of fragment size.
11. Transilluminator and gel documentation system for imaging and recording resolved DNA and RNA.
12. DNA gel electrophoresis equipment.

2.2 Amplification and Digestion of MERS-CoV Fragments

1. Appropriate restriction enzymes and buffers for digestion of each MERS fragment (Table 1).
2. Calf intestinal phosphatase (CIP) for fragment cloning.
3. LB broth supplemented with ampicillin (75 μg/mL) or kanamycin (50 μg/mL) for growth of bacterial cells.
4. Qiaprep Spin Miniprep Kit (Qiagen).
5. Gel electrophoresis-grade agarose.
6. Ethidium bromide, or other DNA-visualizing dye.
7. TAE buffer: 40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA.
8. DNA gel electrophoresis and imaging equipment.
9. Transilluminator and gel documentation system for imaging and recording resolved DNA and RNA.
10. Blue light gel imager (Dark Reader) to extract DNA bands from gel.
11. QIAquick Gel Extraction Kit (Qiagen).
12. 3 M Sodium acetate, pH 5–6.
13. Chloroform.
14. Molecular grade H_2O.

2.3 Assembly and Transcription of Viral Genome

1. NanoDrop (ThermoFisher) or DNA spectrophotometer (BioRad).
2. Cut and purify MERS-A to MERS-F fragments.
3. Fragment with SP6 promoter driving expression of MERS N-gene (used for electroporation negative control and carrier RNA).
4. T4 DNA ligase and ligase buffer (New England BioLabs).
5. Molecular grade H_2O.
6. Chloroform.
7. 70 and 95% ethanol.
8. mMessage mMachine T7 Transcription Kit (Ambion).
9. mMessage mMachine SP6 Transcription Kit (Ambion).

2.4 Cell Preparation

1. Vero-81 cells—approximately 1×10^7 Vero-81 cells or roughly three 80–90% confluent T-175 flasks are necessary for one MERS clone and one control electroporation.
2. Ice-cold molecular grade, RNase free 1× PBS (Gibco).
3. Mammalian cell maintenance medium: 2× DMEM (Gibco), 10% fetal bovine serum (Gibco), 1× antibiotic-antimycotic cocktail (Gibco).
4. 0.25% Trypsin/EDTA (Sigma).

2.5 Electroporation

1. BioRad gene Pulser Excel electroporator or similar device.
2. One 0.4 cm Gene Pulser cuvette per electroporation.
3. Transcribed full-length viral RNA and cells prepared according to Subheadings. 3.3 and 3.4.
4. Virus harvest medium: OptiMEM, 3% HyClone II fetal bovine serum, 1× antibiotic-antimycotic cocktail, 1× nonessential amino acids, 1× sodium pyruvate.

2.6 Cytopathic Effect and Reporter Detection

1. Light microscope with optional fluorescence capacity.

2.7 Confirmation

1. Trizol (Invitrogen)
2. SuperScript II Reverse Transcription kit (Invitrogen)
3. Agarose overlay: Mix agar and media 1:1 prior to overlay.
   (a) SeaKem LE agarose (Lonza)—1.6% agar in ddH_2O.
   (b) 2× MEM—supplemented with 3% fetal bovine serum (Gibco) and 2% antibiotic-antimycotic.
4. 10× Neutral red stain: 0.5% Neutral red, 0.85% NaCl in ddH_2O. Alternately stir and incubate in 55 °C water bath until dissolved, and then filter sterilize. Dilute in 1XPBS for working stock.
5. Light box for ease of plaque visualization and counting.
6. PCR primers designed to the leader and N gene sequence (designed for detection of sgRNA transcripts).

3 Methods

3.1 Transformation and Screening of the Seven MERS-CoV Fragments (BSL1/BSL2 Biocontainment)

1. Thaw each MERS plasmid and chemically competent cells (50 μL aliquots, one tube per plasmid) on ice for 10 min. On ice, add 50 ng (appx. 1 μL) of plasmid to a corresponding labeled tube of competent cells and mix gently. Incubate on ice for 30 min.
2. Heat shock cells in a 42 °C water bath for ~1 min, return to ice, and incubate for 2 min.

3. Add 500 μL of LB broth (without antibiotic) to each competent cell tube. Rock gently at room temperature for 1–6 h.
4. Using a sterile cell spreader, inoculate an appropriate antibiotic selection plate (Table 1) with 50 μL of transformed cells. Incubate for 24–48 h at 28–30 °C (*see* **Note 1**).
5. Pick several colonies per plasmid and transfer into 5 mL of antibiotic-supplemented LB broth (Table 1). Shake at 28–30 °C overnight.
6. Create a library plate from the overnight cultures by spotting 5 μL of each colony culture onto an appropriate antibiotic-supplemented LB plate. Incubate at 28–30 °C overnight, and then store at 4 °C for storage up to 4 weeks.
7. With the remaining bacterial culture volume, perform minipreps (Qiagen Miniprep Kit) on each colony culture to isolate DNA for screening according to the manufacturer's protocol. Elute DNA from the column in 60 μL of Elution Buffer (Qiagen Miniprep kit) heated to 70 °C.
8. Screen colony DNA via restriction digest with appropriate enzymes (*see* Table 1) according to the manufacturer's instructions in a 20 μL reaction volume for 1 h.
9. Resolve digested fragments by gel electrophoresis in a 0.8% agarose gel with ethidium bromide in TAE buffer to validate correct-size fragment. 1 kb DNA ladder or equivalent should be resolved in independent well to validate fragment size. Voltage and time are dependent upon system.
10. Image and document gel for records.
11. Sequence verify colonies with the correct insert prior to advancing to ligation and synthesis of genome-length RNA. Sequencing primers should be spaced approximately 300 nucleotides apart along the CoV fragment of interest. Primers should also be designed to sequence from the plasmid backbone into the inserted CoV fragment.

3.2 Amplification and Digestion of MERS-CoV Fragments (BSL1/BSL2 Biocontainment)

1. Select a replicate from the plasmid library with the correct insert size and sequence for each MERS-CoV fragment (Fig. 2).
2. Inoculate 20 mL LB/antibiotic media from library plate prepared in Subheading 3.1, **step 6**, and shake overnight at 28–30 °C. This should generate enough DNA to assemble two viral clones.
3. Pellet bacterial cells and isolate plasmid DNA using three Qiagen miniprep columns per culture according to the manufacturer's instructions.
4. Elute DNA from the column in 60 μL of elution buffer heated to 70 °C. Combine eluted DNA from each of the three columns in **step 3**, resulting in approximately 180 μL of plasmid DNA per fragment.

5. Digest plasmid DNA with appropriate restriction enzymes (*see* Table 1).
   (a) MERS-A and MERS-F should first be digested with NruI and NotI for 1 h, respectively, according to the manufacturer's instructions (*see* **Note 2**).
   (b) CIP treat fragments by adding 1 unit of CIP per 20 μL of digestion volume and incubate at 37 °C for 1 h.
   (c) For each reaction, extract DNA by mixing 4 volumes of DNA digestion reaction with 1 volume of 3 M sodium acetate and 5 volumes of chloroform. Mix by inversion for 2 min and separate aqueous/organic phases by centrifuging at max speed for 4–5 min at 4 °C.
   (d) Remove the aqueous layer to a clean, labeled tube and add six volumes of isopropyl alcohol. Mix and incubate at room temperature for 10 min.
   (e) Pellet DNA by centrifugation at max speed for 10 min.
   (f) Remove the supernatant, add 1 mL of ice-cold 70% ethanol per tube, and repeat centrifugation.
   (g) Carefully remove the supernatant, add 1 mL of ice-cold 95% ethanol per tube, and repeat centrifugation.
   (h) Carefully remove supernatant and allow DNA pellet to air-dry until no ethanol remains.
   (i) Suspend pellet in molecular grade H_2O heated to 70 °C.
   (j) Continue with digestion of MERS-A and MERS-F, linearized plasmids, with BglI for 1 h according to the manufacturer's instructions.
   (k) Digest plasmids containing MERS fragments B through E with BglI for 1 h according to the manufacturer's instructions.
6. Isolate fragments by gel electrophoresis in a 0.8% agarose gel with ethidium bromide. Allow gel to run until a distinct and easily isolated insert of interest can be seen (*see* **Notes 3** and **4**).
7. Remove the band of interest for each fragment (*see* Table 1).
8. Purify each fragment from the gel using a QIAquick Gel Extraction Kit, according to the manufacturer's instructions. Bands of the same fragment may be pooled and extracted together.
9. Chloroform extract each gel-purified fragment (**steps 5c–i**).

3.3 Assembly and Transcription of Viral Genome (BSL3 Biocontainment)

1. Quantitate the DNA fragments using a DNA spectrophotometer or NanoDrop, and use the base pair length of each fragment to determine the molecule copy number [number of copies = (ng of insert DNA × 6.022 × 10^{23} molecules/mol)/(insert length in base pairs × 1 × 10^9 × 650 g/mol)].

2. Mix an approximately equal copy number of each fragment in a single 1.5 mL Eppendorf tube with a final total volume of 300–400 μL (approx. 20–40 μL per fragment). Add 1 μL 10× ligase buffer and 0.5 μL of ligase per 10 μL of final DNA ligation volume. Incubate the ligation reaction overnight at 4 °C (*see* **Note 5**).
3. Perform a chloroform extraction of the ligation reaction (Subheading 3.2, **steps 5c–i**), resuspending the final DNA pellet in 10–12 μL of DEPC H_2O (*see* **Note 6**).
4. Full-length transcription is performed using the mMessage mMachine T7 transcription kit according to the manufacturer's instructions.
   (a) Combine 10 μL MERS-CoV ligated DNA, 8 μL GTP, 6 μL 10× T7 buffer, 30 μL 2× NTP/CAP, and 6 μL of T7 enzyme.
5. MERS N-gene transcript will be used as a negative control for electroporation and as a carrier RNA co-electroporated with full-length viral RNA transcript generated in **step 4**. MERS N-gene transcript is generated with mMessage mMachine SP6 Transcription Kit according to the manufacturer's instructions (*see* **Note 7**).
   (a) Combine 9 μL template DNA, 9 μL molecular grade H_2O, 6 μL 10× SP6 buffer, 30 μL 2× NTP/CAP, and 6 μL SP6 enzyme.
6. Incubate the transcription reactions at 32 °C for 4–6 h (*see* **Notes 8** and **9**).

3.4 Cell Preparation (BSL2 Biocontainment)

1. Vero-81 cells should be 80–90% confluent on the day of electroporation. Cells are maintained in maintenance medium (*see* Subheading 2.4).
2. To prepare for electroporation, remove media and wash cells in 1× PBS. Add 3 mL of 0.25% trypsin/EDTA (per T-175 flask) and incubate at 37 °C until the cells detach from the flask (*see* **Note 10**).
3. Add 7 mL of maintenance medium per T-175 flask to the trypsinized cells, and then transfer to a 50 mL conical tube. Pellet cells at 2000 × *g* for 5 min at 4 °C (*see* **Notes 6** and **11**).
4. Suspend cells in 10 mL of ice-cold sterile, molecular grade 1× PBS. Count cells using a hemocytometer and pellet cells as in **step 3**. Repeat 1×.
5. Suspend cell pellet in ice-cold sterile 1× PBS to a final concentration of ~10^7 cells/mL.

3.5 Electroporation (BSL3 Biocontainment)

1. Add 60 μL of full-length MERS-CoV RNA (Subheading 3.3, **step 4**) and 20 μL of N-gene transcript (Subheading 3.3, **step 5**) to a microfuge tube. Quickly add 800 μL of ice-cold cell suspension and pipette up and down twice (*see* **Note 12**).

2. For the electroporation negative control use the remainder of N-gene transcript (Subheading 3.3, **step 5**) and add 800 μL of ice-cold Vero-81 cell suspension.
3. Immediately transfer cuvette to a BioRad Gene Pulser Excel electroporator, or a similar device, and set to 50 μF and 450 V. Pulse three times.
4. Remove cuvette and allow to sit at room temperature, without agitation, for 10 min.
5. Transfer electroporated cells to a prepared T-75 flask with 12 mL of harvest medium at 37 °C. Gently rock cells to allow for even distribution. Check for cytopathic effects against the electroporation control over the next 48 h.

3.6 Cytopathic Effect and Reporter Detection (BSL3 Biocontainment)

1. Wild-type MERS-CoV will produce cytopathic effects within 24–72 h (Fig. 3). Syncytia formation results from fusion of neighboring cells via an excess of spike glycoprotein, and can be seen within 12–48 h of electroporation (Fig. 3) (*see* **Note 13**).
2. MERS-CoV can typically be harvested within 48–72 h post-electroporation. Virus harvested just before complete loss of the cellular monolayer will result in 10^7–10^8 PFU of MERS-CoV per mL of culture medium (*see* **Note 14**).
3. If a fluorescent reporter has been inserted in place of an accessory gene open reading frame, then cytopathic effects, viral spread, and confirmation of viral replication can be visualized with a fluorescent microscope (Fig. 5).
4. Clarify viral supernatant by spinning at 3000 × *g* for 15–20 min.
5. Aliquot samples into labeled tubes and store at −80 °C. This viral stock is passage 0 (*see* **Notes 15–17**).

3.7 Confirmation (BSL2 and BSL3 Containment)

1. Virus titration by plaque assay (BSL3 Containment) (Fig. 3):
   (a) MERS-CoV can be titered by plaque assay. 24 h prior to initiating plaque assay seed 3 six-well plates with 5×10^5 Vero-81 cells per well using standard maintenance media.
   (b) Serial dilute virus stocks at 10^{-1} to 10^{-8} in 1× PBS to ensure identification of an appropriate dilution to accurately count plaques.
   (c) Add 200 μL of the appropriate dilution to each of the two wells and incubate at 37 °C for 1 h. Rock plates every 15 min to ensure even distribution of virus inoculum across monolayer.
   (d) Overlay each well with 2 mL of 0.8% agar/media cocktail (*see* Subheading 2) (*see* **Note 18**).
   (e) Plaques are visible and can be stained or picked for plaque purification after 72 h.

(f) To stain, add 2 mL of neutral red stain (*see* Subheading 2) per well and incubate at 37 °C for 2–3 h. Remove neutral red and visualize plaques over a light box to count and titer.

2. Growth curve to assess viral fitness (BSL3 Containment) (Fig. 3):
   (a) Seed a 12-well tissue culture plate with a known number of Vero-81 cells at 24 h prior to infection.
   (b) Infect cells at a low MOI (0.01–0.001).
   (c) Incubate at 37 °C for 1 h rocking plate every 15 min for even distribution of virus inoculum across monolayer.
   (d) Remove supernatant, wash cells with 1× PBS, and add fresh maintenance medium.
   (e) Collect small aliquots of supernatant at time points of interest (0, 4, 8, 12, 24, 48, and 72 h post-infection) to determine viral fitness and growth dynamics.
   (f) Aliquots should be titered via plaque assay as described above (*see* **Note 19**).
3. Verification of viral RNA (Fig. 4):
   (a) Viral RNA can be collected from infected cells, or viral particles, using Trizol, according to the manufacturer's instructions (*see* **Note 20** for handling RNA).
   (b) Reverse transcribe 1 μg of total RNA using a SuperScript II RT kit according to the manufacturer's instructions to generate cDNA.
   (c) Complementary DNA can be verified using PCR designed to identify viral mutants, or sequenced in order to verify viral sequence fidelity.
   (d) PCR of cDNA can also be used to confirm the presence of replication-competent virus by designing a primer to the leader in the 5′ end and an ORF near the 3′ end of the genome, such as that encoding the N gene (*see* **Note 21**).
   (e) Northern blot can likewise be used to identify the presence of CoV sgRNA using the Ambion NorthernMax-Gly kit.
   (f) For detection, design a biotinylated probe against the N gene, located at the 3′ end of the genome, allowing for detection of all viral RNA species present in infected cells. Biotinylated probe should be complementary to the coding strand.

4 Notes

1. Lowering the growth temperature of the bacteria helps to reduce plasmid copy number, thereby increasing plasmid stability in instances where the inserted fragment is toxic.

2. It is important to initially digest, and CIP treat, MERS-A and MERS-F fragments with flanking restriction to prevent inadvertent formation of concatemers and ensure seamless assembly of all fragments.
3. Do not use a UV box to visualize DNA inserts that will be used to create the final full-length DNA genome. UV will introduce random mutations into the inserts that can prevent viral replication and will reduce sequence fidelity. Instead, use a blue-light (dark reader) based transilluminator box to visualize and cut out inserts.
4. Because of the large volume of digested plasmid to be resolved, a gel apparatus should allow for 50–75 μL volumes per well.
5. All steps prior to fragment assembly can be executed under BSL1/BSL2 conditions. MERS-CoV ligation and subsequent steps must be performed in a biosafety level three containment lab.
6. From this point on all working environments should be RNase free. All reagents should be treated with RNase Zap, or similar treatment, to avoid contamination with RNase.
7. Co-electroporation of full-length viral RNA and capped N-gene mRNA has been shown to enhance the transfection efficiency of CoV full-length transcripts.
8. Generally, transcription reactions are performed for a short time at 37 °C. However, in order to ensure the full transcription of all 30 kb of the genome, and to promote sequence fidelity, a lower temperature and longer reaction time are executed.
9. Because of the relative instability of RNA, the full-length transcription reaction should be performed on the same day as the electroporation, typically 5–6 h prior to electroporation.
10. Cells should detach through trypsin digestion and not through physical agitation, as a clean single-cell suspension is desired for efficient electroporation.
11. All subsequent steps should be performed on ice or in a refrigerated centrifuge.
12. Each electroporation cocktail (RNA and cells) should be prepared one at a time. Each subsequent reaction should be prepared following electroporation of the previously prepared cocktail.
13. Successful generation of viral RNA and electroporation will result in loss of cell adherence and cell death within 24–72 h of electroporation. The range of times indicated will depend on the infectious clone being generated.
14. Mutant viruses may take longer to show cytopathic effects and may result in lower overall viral titer. If particularly deleterious

mutations have been introduced, cytopathic effects may not be seen, and a secondary means of confirmation will be required to assess the success of virus generation. Additional means of confirmation may include staining with antibody and visualizing by immunofluorescence microscopy or isolating total RNA from cell lysates to confirm by RT-PCR.

15. If deleterious mutations have been introduced during cloning, be sure to sequence verify passage 0 and subsequent virus passages, preferentially using P0 virus to assure sequence fidelity.
16. Expanding viral stocks by passage through cell culture can lead to the natural accumulation of tissue culture-adapted mutations. Viral stocks should routinely be sequence validated.
17. Freezing and thawing virus will result in an approximate log drop in titer for each freeze-thaw cycle.
18. Plates should not be disturbed (i.e., moved) during incubation to ensure clear identification of plaques.
19. It is recommended to run wild-type virus and a non-mutated infectious clone virus as controls for a growth curve of any reverse genetics mutant.
20. At the time of writing this chapter MERS-CoV genomic length RNA is not considered a select agent. Therefore, samples can be inactivated in Trizol under BSL3 conditions, and then moved to BSL2 for subsequent work. In the case of select agents, like SARS-CoV, the handling of genomic length RNA falls under the Federal Select Agent Program, and must be handled accordingly.
21. During replication, CoVs generate a nested set of sgRNA intermediates containing the leader sequence at the 5′end, a portion of the ORFs present in the CoV genome, and a 3′ UTR (Fig. 4). As a result, primers built into the leader sequence and the N-gene will generate PCR products of various lengths representing the cDNA from both full-length and sgRNA. If the presence of sgRNA is detected, it can be assumed that the CoV clone is infectious and replication competent.

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Chapter 6

Reverse Genetics System for the Avian Coronavirus Infectious Bronchitis Virus

Erica Bickerton, Sarah M. Keep, and Paul Britton

Abstract

We have developed a reverse genetics system for the avian coronavirus infectious bronchitis virus (IBV) in which a full-length cDNA corresponding to the IBV genome is inserted into the vaccinia virus genome under the control of a T7 promoter sequence. Vaccinia virus as a vector for the full-length IBV cDNA has the advantage that modifications can be introduced into the IBV cDNA using homologous recombination, a method frequently used to insert and delete sequences from the vaccinia virus genome. Here, we describe the use of transient dominant selection as a method for introducing modifications into the IBV cDNA that has been successfully used for the substitution of specific nucleotides, deletion of genomic regions, and exchange of complete genes. Infectious recombinant IBVs are generated in situ following the transfection of vaccinia virus DNA, containing the modified IBV cDNA, into cells infected with a recombinant fowlpox virus expressing T7 DNA-dependant RNA polymerase.

Key words Transient dominant selection (TDS), Vaccinia virus, Infectious bronchitis virus (IBV), Coronavirus, Avian, Reverse genetics, Nidovirus, Fowlpox virus, T7 RNA polymerase

1 Introduction

The *Coronaviridae* form part of the order *Nidovirales*, which comprises two subfamilies, the *Coronavirinae* and *Torovirinae*. There are four genera of coronaviruses, *alpha-*, *beta-*, *gamma-*, and *delta-coronaviruses* [1], which were so named for their visual resemblance to the corona of the sun in negatively stained preparations [2]. Coronaviruses are enveloped viruses with a single-stranded positive-sense RNA genome of 26–32 kb, the largest genomes of all RNA viruses currently known, that replicate in the cytoplasm of infected cells. The genome associates with the nucleoprotein (N) forming a helical nucleocapsid within the virus particles. Although common among negative-sense RNA viruses, coronaviruses are the only positive-sense RNA viruses to possess helical nucleocapsids, which are enclosed within lipid envelopes containing the spike (S) glycoprotein, membrane (M) protein, and envelope (E) protein.

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Avian infectious bronchitis virus (IBV) is a *gammacoronavirus* that is the etiological agent of infectious bronchitis (IB), an acute and highly contagious disease of poultry characterized by nasal discharge, snicking, tracheal ciliostasis, and rales [3]. IBV replicates primarily in the respiratory tract but also in many other epithelial surfaces including oviducts, enteric surfaces, and kidneys [4–7]. Following infection with IBV, egg production and quality may be impaired in layers and weight gain in broilers is reduced [8]. Infected birds are predisposed to secondary bacterial infections such as *colibacillosis* and mortality in young chicks is not uncommon. Fecal excretion of the virus is a consequence of replication in the intestinal tract; however, this does not normally result in clinical disease.

Infectious bronchitis was first described in the USA in the 1930s [9–11] and is prevalent in poultry farming across the world due to the intensive nature of poultry production, estimated to involve the global production of 55 billion chickens (50 billion broilers and five billion layers) on an annual basis. According to a new market research report published by MarketsandMarkets, the global poultry meat market was worth $179,551.2 million in 2012 and is expected to reach $250,949.5 million by 2018. In a report, commissioned by Defra in 2005 [12], IBV was indicated as a major cause of ill health among chickens and was implicated as being responsible for more economic loss in the UK poultry industry than any other disease [13, 14]; IBV was estimated to cost the UK economy nearly £19 million per year, mainly due to loss of egg production, with serious implications for animal welfare. The cost of control through vaccination is approximately £5 million per year in the UK.

Coronaviruses are enveloped viruses that replicate in the cell cytoplasm. Coronavirus genomes consist of single-stranded positive-sense RNA, and are the largest of all the RNA viruses ranging from approximately 27–32 kb; the genome of IBV is 27.6 kb. Molecular analysis of the role of individual genes in the pathogenesis of RNA viruses has been advanced by the availability of full-length cDNAs, for the generation of infectious RNA transcripts that can replicate and result in infectious viruses. The assembly of full-length coronavirus cDNAs was hampered due to regions from the replicase gene being unstable in bacteria. We therefore devised a reverse genetics strategy for IBV involving the insertion of a full-length cDNA copy of the IBV genome, under the control of a T7 RNA promoter, into the vaccinia virus genome in place of the thymidine kinase (TK) gene. A hepatitis δ ribozyme (HδR) is located downstream of the coronavirus poly(A) tail followed by a T7 termination sequence. IBV infectious RNA is generated from the T7 promoter immediately adjacent to the 5′ end of the IBV cDNA using T7 RNA polymerase and terminates at the T7 termination sequence downstream of the HδR sequence, which autocleaves itself and the T7 termination sequence at the end of the poly(A) sequence, resulting in an authentic IBV genomic RNA

copy. Infectious IBV is recovered in situ in cells both transfected with vaccinia virus DNA and infected with a recombinant fowlpox virus expressing T7 RNA polymerase [15].

One of the main advantages of using vaccinia virus as a vector for IBV cDNA is its ability to accept large quantities of foreign DNA without loss of integrity and stability [16]. A second and equally important advantage is the ability to modify the IBV cDNA within the vaccinia virus vector through transient dominant selection (TDS), a method taking advantage of recombinant events between homologous sequences [17, 18]. The TDS method relies on a three-step procedure. In the first step, the modified IBV cDNA is inserted into a plasmid containing a selective marker under the control of a vaccinia virus promoter. In our case we use a plasmid, pGPTNEB193 (Fig. 1; [19]), which contains a dominant selective marker gene, *Escherichia coli guanine phosphoribosyltransferase* (*Ecogpt*; [20]), under the control of the vaccinia virus P7.5K early/late promoter.

In the second step, this complete plasmid sequence is integrated into the IBV sequence within the vaccinia virus genome (Fig. 2). This occurs as a result of a single crossover event involving homologous recombination between the IBV cDNA in the plasmid and the IBV cDNA sequence in the vaccinia virus genome. The resulting recombinant vaccinia viruses (rVV) are highly unstable due to the presence of duplicate sequences and are only maintained by the selective pressure of the *Ecogpt* gene, which confers resistance to

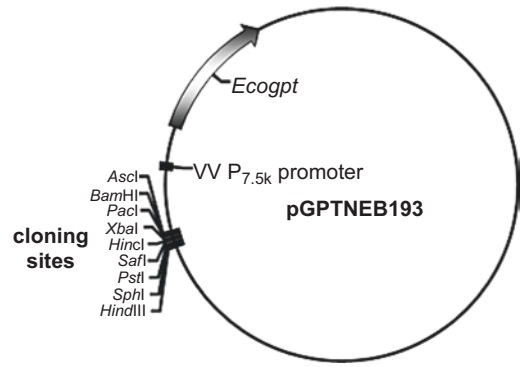

Fig. 1 Schematic diagram of the recombination vector for insertion of genes into a vaccinia virus genome using TDS. Plasmid pGPTNEB193 contains the *Ecogpt* selection gene under the control of the vaccinia virus early/late $P_{7.5K}$ promoter, a multiple cloning region for the insertion of the sequence to be incorporated into the vaccinia virus genome, and the *bla* gene (not shown) for ampicillin selection of the plasmid in *E. coli*. For modification of the IBV genome, a sequence corresponding to the region being modified, plus flanking regions of 500–800 nucleotides for recombination purposes, is inserted into the multiple cloning sites using an appropriate restriction endonuclease. The plasmid is purified from *E. coli* and transfected into Vero cells previously infected with a recombinant vaccinia virus containing a full-length cDNA copy of the IBV genome

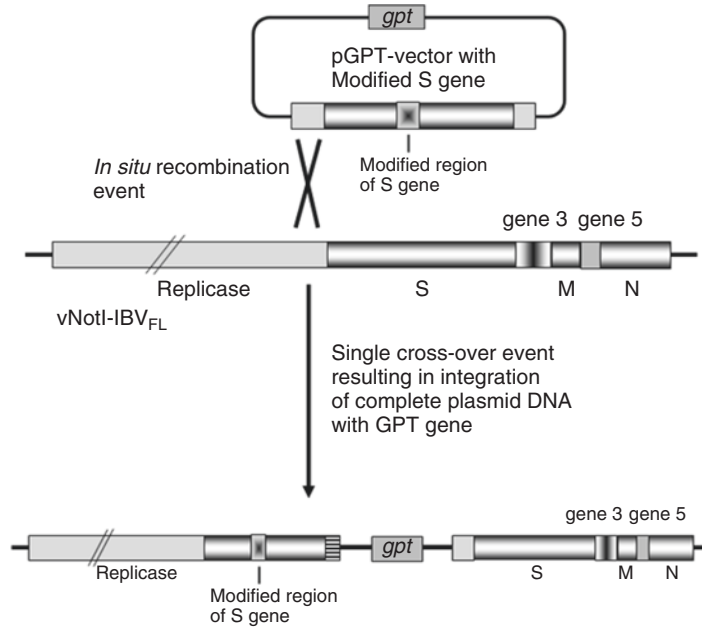

Fig. 2 Schematic diagram demonstrating the TDS method for integrating a modified IBV sequence into the full-length IBV cDNA within the genome of a recombinant vaccinia virus (vNotI-IBVFL). The diagram shows a potential first single-step recombination event between the modified IBV sequence within pGPTNEB193 and the IBV cDNA within vNotI-IBVFL. In order to guarantee a single-step recombination event any potential recombinant vaccinia viruses are selected in the presence of MPA; only vaccinia viruses expressing the *Ecogpt* gene are selected. The main IBV genes are indicated, the replicase, spike (S), membrane (M), and nucleocapsid (N) genes. The IBV gene 3 and 5 clusters that express three and two gene products, respectively, are also indicated. In the example shown a modified region of the S gene is being introduced into the IBV genome

mycophenolic acid (MPA) in the presence of xanthine and hypoxanthine [17]. In the third step, the MPA-resistant rVVs are grown in the absence of MPA selection, resulting in the loss of the *Ecogpt* gene due to a second single homologous recombination event between the duplicated sequences (Fig. 3). During this third step two recombination events can occur; one event will result in the generation of the original (unmodified) IBV sequence and the other in the generation of an IBV cDNA containing the desired modification (i.e., the modification within the plasmid sequence). In theory these two events will occur at equal frequency; however in practice this is not necessarily the case.

To recover infectious rIBVs from the rVV vector, rVV DNA is transfected into primary chick kidney (CK) cells previously infected with a recombinant fowlpox virus expressing T7 RNA polymerase (rFPV-T7; [21]). In addition, a plasmid, pCi-Nuc [15, 22], expressing the IBV nucleoprotein (N), under the control of both the cytomegalovirus (CMV) RNA polymerase II promoter and the T7 RNA

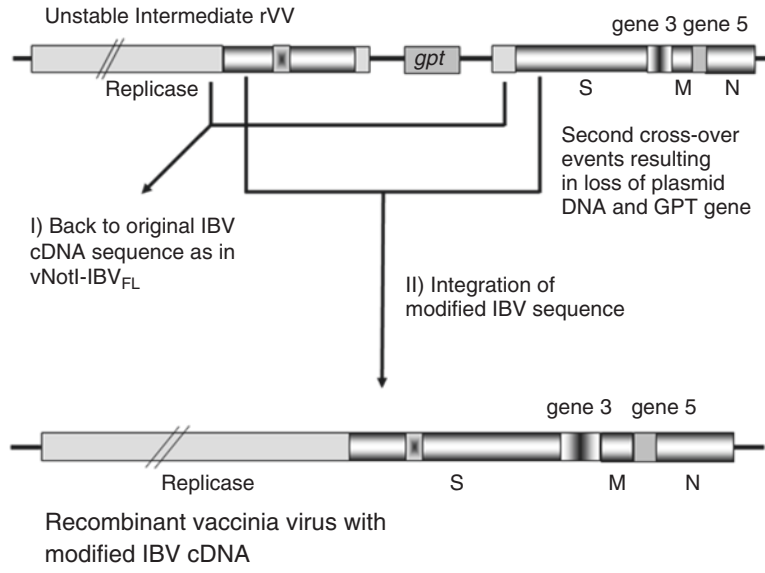

Fig. 3 Schematic diagram demonstrating the second step of the TDS method. Integration of the complete pGPTNEB193 plasmid into the vaccinia virus genome results in an unstable intermediate because of the presence of tandem repeat sequences, in this example the 3′ end of the replicase gene, the S gene, and the 5′ end of gene 3. The second single-step recombination event is induced in the absence of MPA; loss of selection allows the unstable intermediate to lose one of the tandem repeat sequences including the *Ecogpt* gene. The second step recombination event can result in either (*I*) the original sequence of the input vaccinia virus IBV cDNA sequence, in this case shown as a recombination event between the two copies of the 3′ end of the replicase gene which results in loss of the modified S gene sequence along with *Ecogpt* gene, or (*II*) retention of the modified S gene sequence and loss of the original S gene sequence and *Ecogpt* gene as a result of a potential recombination event between the two copies of the 5′ end of the S gene sequence. This event results in a modified S gene sequence within the IBV cDNA in a recombinant vaccinia virus

promoter, is co-transfected into the CK cells. Expression of T7 RNA polymerase in the presence of the IBV N protein and the rVV DNA, containing the full-length IBV cDNA under the control of a T7 promoter, results in the generation of infectious IBV RNA, which in turn results in the production of infectious rIBVs (Fig. 4). Primary CK cells are refractory for growth of most IBV isolates; therefore rIBVs expressing S glycoproteins from such isolates cannot be recovered using CK cells. In order to recover such rIBVs, the supernatants from the transfected CK cells are used to infect 10-day-old embryonated hen's eggs. Allantoic fluid is collected and any potential virus passed a further three times in 10-day-old embryos. RNA is extracted from the allantoic fluid of infected eggs and RT-PCR followed by sequencing is used to confirm the identity of the rIBV.

The overall procedure is a multistep process which can be divided into two parts: the generation of an rVV containing the

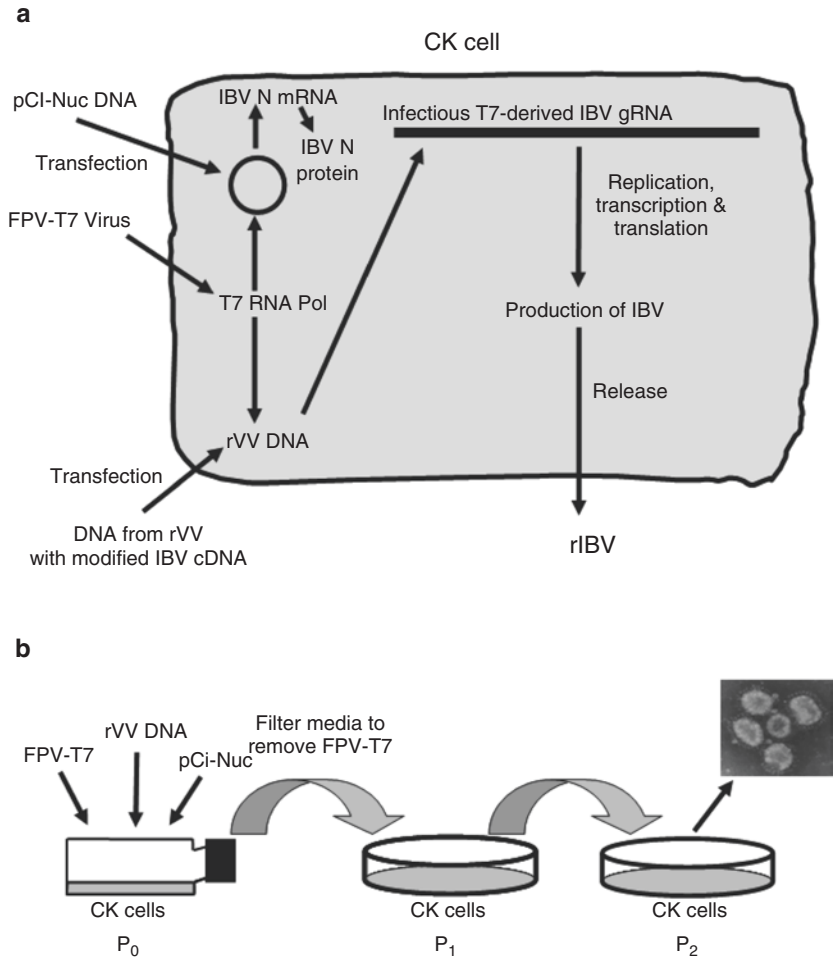

Fig. 4 A schematic representation of the recovery process for obtaining rIBV from DNA isolated from a recombinant vaccinia virus containing a full-length IBV cDNA under the control of a T7 promoter. (**a**) In addition to the vaccinia virus DNA containing the full-length IBV cDNA under the control of a T7 promoter a plasmid, pCI-Nuc, expressing the IBV nucleoprotein, required for successful rescue of IBV, is transfected into CK cells previously infected with a recombinant fowlpox virus, FPV-T7, expressing T7 RNA polymerase. The T7 RNA polymerase results in the synthesis of an infectious RNA from the vaccinia virus DNA that consequently leads to the generation of infectious IBV being released from the cell. (**b**) Any recovered rIBV present in the media of P_0 CK cells is used to infect P_1 CK cells. The media is filtered through a 0.22 μm filter to remove any FPV-T7 virus. IBV-induced CPE is normally observed in the P_1 CK cells following a successful recovery experiment. Any rIBV is passaged a further two times, P_2 and P_3, in CK cells. Total RNA is extracted from the P_1–P_3 CK cells and the IBV-derived RNA analyzed by RT-PCR for the presence of the required modification

modified IBV cDNA (Fig. 5) and the recovery of infectious rIBV from the rVV vector (Fig. 4). The generation of the *Ecogpt* plasmids, based on pGPTNEB193, containing the modified IBV cDNA, is by standard *E. coli* cloning methods [23, 24] and is not described here. General methods for growing vaccinia virus have been published by Mackett et al. [25] and for using the TDS method for modifying the vaccinia virus genome by Smith [26].

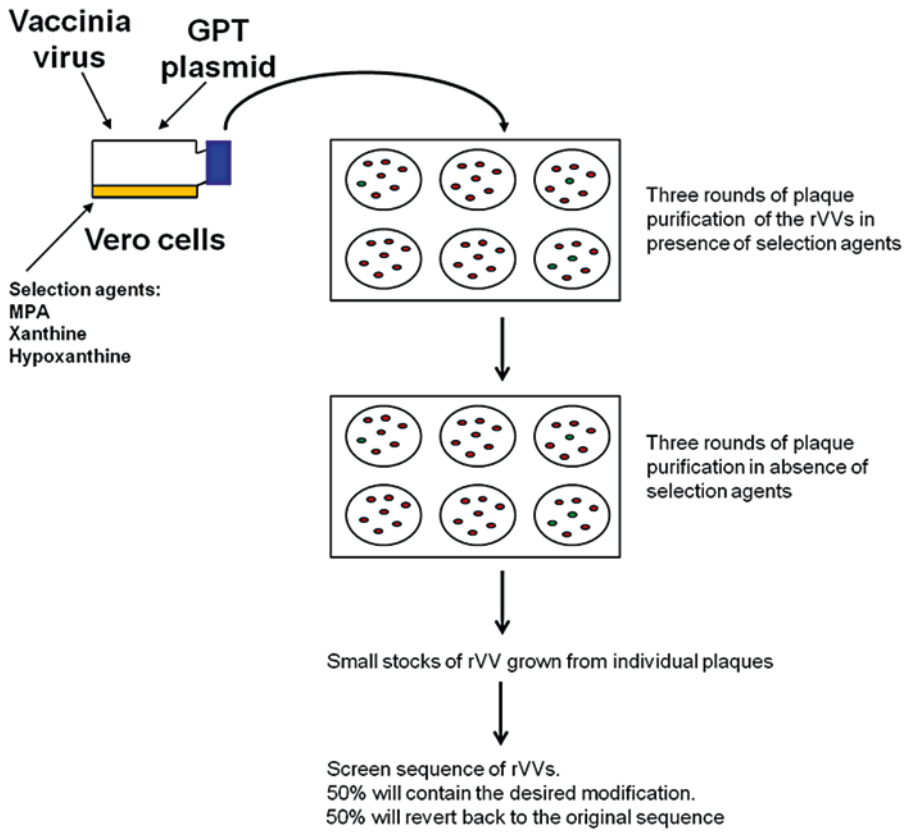

Fig. 5 Schematic detailing the multistep process of constructing a recombinant vaccinia virus. Vero cells are infected with rVV containing IBV cDNA and then transfected with a plasmid containing the IBV sequence to be inserted and the selective marker gene *Ecogpt*. Homologous recombination occurs and the complete plasmid sequence is inserted into the rVV. The *Ecogpt* gene allows positive selection of these rVV as it confers resistance to MPA in the presence of xanthine and hypoxanthine. The viruses are plaque purified three times in the presence of selection agents ensuring that no wild-type VV is present. The removal of the selection agents results in a second recombination event with the loss of the *Ecogpt* gene. Plaque purification in the absence of selection agents ensures not only the loss of the GPT gene but also the maintenance of a single viral population. Small stocks of rVV are grown from individual plaques which are screened through PCR for the desired modification; this is found in theoretically 50% of rVVs

2 Materials

2.1 Homologous Recombination and Transient Dominant Selection in Vero Cells

1. Vero cells.
2. PBSa: 172 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, adjusted to pH 7.2 with HCl.
3. 1× Eagle's Minimum Essential Medium (E-MEM) with Earle's salts, 2 mM L-glutamine, and 2.2 g/L sodium bicarbonate.
4. BES medium: 1× E-MEM, 0.3% tryptose phosphate broth (TPB), 0.2% bovine serum albumin (BSA), 20 mM N, N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 0.21% sodium bicarbonate, 2 mM L-glutamine, 250 U/mL nystatin, 100 U/mL penicillin, and 100 U/mL streptomycin.

5. OPTIMEM 1 with GLUTAMAX-1 (Life Technologies).
6. Lipofectin (Life Technologies).
7. Mycophenolic acid (MPA): 10 mg/mL in 0.1 M NaOH (30 mM); 400× concentrated.
8. Xanthine: 10 mg/mL in 0.1 M NaOH (66 mM); 40× concentrated. Heat at 37 °C to dissolve.
9. Hypoxanthine: 10 mg/mL in 0.1 M NaOH (73 mM); 667× concentrated.
10. Screw-top 1.5 mL microfuge tubes with gasket.
11. Cup-form sonicator.
12. 2× E-MEM: 2× E-MEM, 10% fetal calf serum, 0.35% sodium bicarbonate, 4 mM L-glutamine, 1000 U/mL nystatin, 200 U/mL penicillin, and 200 U/mL streptomycin.
13. 2% Agar.
14. *Ecogpt* selection medium: 1× E-MEM, 75 μM MPA, 1.65 mM xanthine, 109 μM hypoxanthine, 1% agar (*see* **Note 7**).
15. Overlay medium: 1× E-MEM, 1% agar.
16. 1% Neutral red solution (H_2O).

2.2 Extraction of DNA from Recombinant Vaccinia Virus

1. 20 mg/mL Proteinase K.
2. 2× Proteinase K buffer: 200 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.4% SDS, 400 mM NaCl.
3. Phenol/chloroform/isoamyl alcohol (25:24:1).
4. Chloroform.
5. Absolute ethanol.
6. 70% Ethanol.
7. QlAamp DNA mini kit (QIAGEN).
8. 3 M Sodium acetate.

2.3 Production of Large Stocks of Vaccinia Virus

1. BHK-21 maintenance medium: Glasgow-Modified Eagle's Medium (G-MEM), 2 mM L-glutamine, 0.275% sodium bicarbonate, 1% fetal calf serum, 0.3% TPB, 500 U/mL nystatin, 100 U/mL penicillin, and 100 U/mL streptomycin.
2. TE buffer: 10 mM Tris–HCl pH 9, 1 mM EDTA.
3. BHK-21 cells.
4. 50 mL Falcon tubes.

2.4 Vaccinia Virus Partial Purification

1. 30% Sucrose (w/v) in 1 mM Tris–HCl pH 9, filtered through 0.22 μm.
2. Superspin 630 rotor and Sorvall OTD65B ultracentrifuge or equivalent.

2.5 Analysis of Vaccinia Virus DNA by Pulsed Field Agarose Gel Electrophoresis

1. 10× TBE buffer: 1 M Tris–HCl, 0.9 M boric acid pH 8, and 10 mM EDTA.
2. Pulsed field-certified ultrapure DNA-grade agarose.
3. DNA markers (e.g., 8–48 kb markers, Biorad).
4. 0.5 mg/mL Ethidium bromide.
5. CHEF-DR® II pulsed field gel electrophoresis (PFGE) apparatus (Biorad) or equivalent.
6. 6× Sample loading buffer: 62.5% Glycerol, 62.5 mM Tris–HCl pH 8, 125 mM EDTA, and 0.06% bromophenol blue.

2.6 Preparation of rFPV-T7 Stock Virus

1. Chicken embryo fibroblast (CEF) cells.
2. CEF maintenance medium: 1× 199 Medium with Earle's Salts, 0.3% TPB, 2% newborn calf serum (NBCS), 0.225% sodium bicarbonate, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 500 U/mL nystatin.

2.7 Recovery of rIBV and Serial Passage on CK Cells

1. Chick kidney (CK) cells.
2. Stock of rFPV-T7 virus.
3. The rVV DNA prepared from large partially purified stocks of rVV.
4. Plasmid pCi-Nuc which contains IBV nucleoprotein under the control of the CMV and T7 promoters.
5. 0.22 μm Syringe-driven filters.
6. 5 mL Syringes.

3 Methods

3.1 Infection/ Transfection of Vero Cells with Vaccinia Virus

1. Freeze-thaw the vaccinia virus containing the full-length IBV cDNA genome to be modified three times (37 °C/dry ice) and sonicate for 2 min using a cup-form sonicator, continuous pulse at 70% duty cycle, 7 output control (*see* **Notes 1–4**).
2. Infect 6-well plates of 40% confluent monolayers of Vero cells with the rVV at a multiplicity of infection (MOI) of 0.2. Use two independent wells per recombination (*see* **Notes 1–4**).
3. Incubate at 37 °C 5% CO_2 for 2 h to allow the virus to infect the cells.
4. After 1 h of incubation, prepare the following solutions for transfection:

   Solution A: For each transfection: Dilute 5 μg of modified pGPTNEB193 (containing the modified IBV cDNA) in 1.5 mL of OPTIMEM medium.

   Solution B: Dilute 12 μL of lipofectin in 1.5 mL of OPTIMEM for each transfection.

5. Incubate solutions A and B separately for 30 min at room temperature, then mix the two solutions together, and incubate the mixture at room temperature for 15 min.
6. During the 15-min incubation, remove the inoculum from the vaccinia virus-infected cells and wash the cells twice with OPTIMEM.
7. Add 3 mL of the transfection mixture (prepared in **step 5**) to each well.
8. Incubate for 60–90 min at 37 °C 5% CO_2 (*see* **Note 5**).
9. Remove the transfection mixture from each well and replace it with 5 mL of BES medium.
10. Incubate the transfected cells overnight at 37 °C, 5% CO_2.
11. The following morning add the MXH selection components, MPA 12.5 μL, xanthine 125 μL, and hypoxanthine 7.4 μL, directly to each well (*see* **Note 6**).
12. Incubate the cells at 37 °C 5% CO_2 until they display extensive vaccinia virus-induced cytopathic effect (CPE) (normally 2 days).
13. Harvest the infected/transfected cells into the cell medium of the wells and centrifuge for 3–4 min at 300 × *g*. Discard supernatant, resuspend the pellet in 400 μL 1× E-MEM, and store at −20 °C.

3.2 Plaque Purification in the Presence of GPT Selection Agents: Selection of MPA-Resistant Recombinant Vaccinia Viruses (GPT+ Phenotype)

1. Freeze-thaw the vaccinia virus produced from Subheading 3.1 three times and sonicate as described in the previous subheading (Subheading 3.1, **step 1**).
2. Remove the medium from confluent Vero cells in 6-well plates and wash the cells once with PBSa.
3. Prepare 10^{-1} to 10^{-3} serial dilutions of the recombinant vaccinia virus in 1× E-MEM.
4. Remove the PBSa from the Vero cells and add 500 μL of the diluted virus per well.
5. Incubate for 1–2 h at 37 °C 5% CO_2.
6. Remove the inoculum and add 3 mL of the *Ecogpt* selection medium (*see* **Note 7**).
7. Incubate for 3–4 days at 37 °C 5% CO_2 and stain the cells by adding 2 mL of 1× E-MEM containing 1% agar and 0.01% neutral red.
8. Incubate the cells at 37 °C 5% CO_2 for 6–24 h and pick 2–3 well-isolated plaques for each recombinant, by taking a plug of agarose directly above the plaque. Place the plug of agar in 400 μL of 1× E-MEM.
9. Perform two further rounds of plaque purification for each selected recombinant vaccinia virus in the presence of *Ecogpt* selection medium, as described in **steps 1–8** (*see* **Note 8**).

3.3 Plaque Purification in the Absence of GPT Selection Agents: Selection of MPA-Sensitive Recombinant Vaccinia Viruses (Loss of GPT$^+$ Phenotype)

1. Take the MPA-resistant plaque-purified rVVs which have been plaque purified a total of three times as described in Subheading 3.2 and freeze-thaw and sonicate as described in Subheading 3.1, **step 1**.
2. Remove the medium from confluent Vero cells in 6-well plates and wash the cells with PBSa.
3. Prepare 10^{-1} to 10^{-3} serial dilutions of the recombinant vaccinia virus in 1× E-MEM.
4. Remove the PBSa from the Vero cells and add 500 µL of the diluted virus per well.
5. Incubate for 1–2 h at 37 °C 5% CO_2.
6. Remove the inoculum and add 3 mL of the overlay medium (*see* **Note 9**).
7. Incubate for 3–4 days at 37 °C 5% CO_2 and stain the cells by adding 2 mL 1× E-MEM containing 1% agar and 0.01% neutral red.
8. Incubate the cells at 37 °C 5% CO_2 for 6–24 h and pick 3–6 well-isolated plaques for each recombinant, by taking a plug of agar directly above the plaque. Place the plug of agar in 400 µL of 1× E-MEM (*see* **Note 8**).
9. Perform two further rounds of plaque purification for each selected recombinant vaccinia virus in the presence of selection medium, as described in **steps 1–8**.

3.4 Production of Small Stocks of Recombinant Vaccinia Viruses

1. Take the MPA-sensitive plaque-purified rVVs which have been plaque purified a total of three times as described in Subheading 3.3 and freeze-thaw and sonicate as described in Subheading 3.1, **step 1**.
2. Remove the medium from confluent Vero cells in six-well plates and wash the cells with PBSa.
3. Dilute 150 µL of the sonicated rVVs in 350 µL of BES medium.
4. Remove the PBSa from the Vero cells and add 500 µL of the diluted rVVs per well.
5. Incubate at 37 °C and 5% CO_2 for 1–2 h.
6. Add 2.5 mL per well of BES medium.
7. Incubate the infected Vero cells at 37 °C and 5% CO_2 until the cells show signs of extensive vaccinia virus-induced CPE (approx. 4 days).
8. Scrape the Vero cells into the medium, and harvest into 1.5 mL screw-cap tubes with gaskets.
9. Centrifuge for 3 min at 16,000 × *g* in a benchtop centrifuge.
10. Discard the supernatants, resuspend the cells in a total of 400 µL of BES cell culture medium, and store at −20 °C.

3.5 DNA Extraction from Small Stocks of Recombinant Vaccinia Virus for Screening by PCR

There are two methods for DNA extraction:

1. DNA extraction using phenol/chloroform/isoamyl alcohol.
   (a) To 100 μL of rVV stock produced in Subheading 3.4, add 100 μL 2× proteinase K buffer and 2 μL of the proteinase K stock. Gently mix and incubate at 50 °C for 2 h.
   (b) Add 200 μL of phenol/chloroform/isoamyl alcohol to the proteinase K-treated samples, mix by inverting the tube 5–10 times, and centrifuge at 16,000 × *g* for 5 min (*see* **Note 10**).
   (c) Take the upper aqueous phase and repeat **step 2** twice more.
   (d) Add 200 μL of chloroform to the upper phase and mix and centrifuge as in **step 2**.
   (e) Take the upper phase and precipitate the vaccinia virus DNA by adding 2.5 volumes of absolute ethanol; the precipitated DNA should be visible. Centrifuge the precipitated DNA at 16,000 × *g* for 20 min. Discard the supernatant.
   (f) Wash the pelleted DNA with 400 μL 70% ethanol and centrifuge at 16,000 × *g* for 10 min. Discard the supernatant, carefully, and remove the last drops of 70% ethanol using a capillary tip.
   (g) Resuspend the DNA in 30 μL water and store at 4 °C (*see* **Note 11**).
2. Extraction of rVV DNA using the Qiagen QlAamp DNA mini kit.
   (a) Follow the blood/bodily fluid spin protocol and start with 200 μL of rVV stock produced in Subheading 3.4.
   (b) Elute the rVV DNA in 200 μL buffer AE (provided in the kit) and store at 4 °C.

At this stage the extracted rVV DNA is analyzed by PCR and/or sequence analysis for the presence/absence of the *Ecogpt* gene and for the modifications within the IBV cDNA sequence. Once an rVV is identified that both has lost the *Ecogpt* gene and contains the desired IBV modification, large stocks are produced. Typically two rVVs will be taken forward at this stage, which ideally have been generated from different wells of the infection/transfection of Vero cells stage previously described in Subheading 3.1. Once the large stocks of the chosen rVVs have been produced, rVV DNA will be extracted and prepared for the recovery of rIBV.

3.6 Production of Large Stocks of Vaccinia Virus

1. Freeze-thaw and sonicate the chosen rVV stocks from Subheading 3.4 as described in Subheading 3.1, **step 1**.
2. Dilute the sonicated virus in BHK-21 maintenance medium and infect 11× T150 flasks of confluent monolayers of BHK-21

cells using 2 mL of the diluted vaccinia virus per flask at an MOI of 0.1–1.

3. Incubate the infected cells for 1 h at 37 °C and 5% CO_2.
4. Add 18 mL of pre-warmed (37 °C) BHK-21 maintenance medium and incubate the infected cells at 37 °C and 5% CO_2 until the cells show an advanced CPE (normally about 2–3 days postinfection). At this stage the cells should easily detach from the plastic.
5. Either continue to **step 6** or freeze the flasks in plastic boxes lined with absorbent material and labeled with biohazard tape at −20 °C until further use.
6. If prepared from frozen, the flasks need to be defrosted by leaving them at room temperature for 15 min and then at 37 °C until the medium over the cells has thawed.
7. Tap the flasks to detach the cells from the plastic; if necessary use a cell scraper.
8. Transfer the medium containing the cells to 50 mL Falcon tubes and centrifuge at 750 × *g* for 15 min at 4 °C to pellet the cells.
9. Discard the supernatant (99% of vaccinia virus is cell associated) and resuspend the cells in 1 mL of TE buffer per flask.
10. Pool the resuspended cells, then aliquot into screw-top microfuge tubes with gasket, and store at −70 °C.
11. Use one 1 mL aliquot of the resuspended cells as a virus stock. Use the resuspended cells from the remaining 10 flasks for partial purification.

3.7 Vaccinia Virus Partial Purification

1. Freeze-thaw and sonicate the resuspended cells generated from Subheading 3.6 as described in Subheading 3.1, **step 1**.
2. Centrifuge at 750 × *g* for 10 min at 4 °C to remove the cell nuclei.
3. Keep the supernatant and add TE buffer to give a final volume of 13 mL.
4. Add 16 mL of the 30% sucrose solution into a Beckman ultra-clear (25 × 89 mm) ultracentrifuge tube and carefully layer 13 mL of the cell lysate from **step 3** on to the sucrose cushion.
5. Centrifuge the samples using an ultracentrifuge at 36,000 × *g*, 4 °C for 60 min.
6. The partially purified vaccinia virus particles form a pellet under the sucrose cushion. After centrifugation, carefully remove the top layer (usually pink) and the sucrose layer with a pipette. Wipe the sides of the tube carefully with a tissue to remove any sucrose solution.
7. Resuspend each pellet in 5 mL TE buffer and store at −70 °C.

3.8 Extraction of Vaccinia Virus DNA from Large Partially Purified rVV Stocks

1. Defrost the partially purified vaccinia virus from Subheading 3.7 at 37 °C.
2. Add 5 mL of pre-warmed 2× proteinase K buffer and 100 μL of 20 mg/mL proteinase K to the partially purified vaccinia virus in a 50 mL Falcon tube. Incubate at 50 °C for 2.5 h (*see* **Notes 1–4**).
3. Transfer into a clean 50 mL Falcon tube.
4. Add 5 mL of phenol/chloroform/isoamyl alcohol, mix by inverting the tube 5–10 times, and centrifuge at 1100 × *g* in a benchtop centrifuge for 15 min at 4 °C. Transfer the upper phase to a clean 50 mL Falcon tube using wide-bore pipette tips (*see* **Note 10–11**).
5. Repeat **step 3**.
6. Add 5 mL chloroform, mix by inverting the tube 5–10 times, and centrifuge at 1100 × *g* for 15 min at 4 °C. Transfer the upper phase into a clean 50 mL Falcon tube.
7. Precipitate the vaccinia virus DNA by adding 2.5 volumes of −20 °C absolute ethanol and 0.1 volumes of 3 M sodium acetate. Centrifuge at 1200 × *g*, 4 °C for 60–90 min. A glassy pellet should be visible.
8. Discard the supernatant and wash the DNA using 10 mL −20 °C 70% ethanol. Leave on ice for 5 min and centrifuge at 1200 × *g*, 4 °C for 30–45 min. Discard the supernatant and remove the last drops of ethanol using a capillary tip. Dry the inside of the tube using a tissue to remove any ethanol.
9. Air-dry the pellet for 5–10 min.
10. Resuspend the vaccinia DNA in 100 μL of water. Do not pipette to resuspend as shearing of the DNA will occur.
11. Leave the tubes at 4 °C overnight. If the pellet has not dissolved totally, add more water.
12. Measure the concentration of the extracted DNA using a nanodrop or equivalent.
13. Store the vaccinia virus DNA at 4 °C. DO NOT FREEZE (*see* **Note 6**).

3.9 Analysis of Vaccinia Virus DNA by Pulsed Field Agarose Gel Electrophoresis (PFGE)

1. Prepare 2 L of 0.5× TBE buffer for preparation of the agarose gel and as electrophoresis running buffer; 100 mL is required for a 12.7 × 14 cm agarose gel and the remainder is required as running buffer.
2. Calculate the concentration of agarose that is needed to analyze the range of DNA fragments to be analyzed. Increasing the agarose concentration decreases the DNA mobility within the gel, requiring a longer run time or a higher voltage. However, a higher voltage can increase DNA degradation and

reduce resolution. A 0.8% agarose gel is suitable for separating DNA ranging between 50 and 95 kb. A 1% agarose gel is suitable for separating DNA ranging between 20 and 300 kb.

3. Place the required amount of agarose in 100 mL 0.5× TBE buffer and microwave until the agarose is dissolved. Cool to approximately 50–60 °C.
4. Clean the gel frame and comb with MQ water followed by 70% ethanol. Place the gel frame on a level surface, assemble the comb, and pour the cooled agarose into the gel frame. Remove any bubbles using a pipette tip, allow the agarose to set (approx. 30–40 min), and store in the fridge until required.
5. Place the remaining 0.5× TBE buffer into the CHEF-DR® II PFGE electrophoresis tank and switch the cooling unit on. Leave the buffer circulating to cool.
6. Digest 1 μg of the DNA with a suitable restriction enzyme such as Sal I in a 20 μL reaction.
7. Add the sample-loading dye to the digested vaccinia virus DNA samples and incubate at 65 °C for 10 min.
8. Place the agarose gel in the electrophoresis chamber; load the samples using wide-bore tips and appropriate DNA markers (*see* **Note 11**).
9. The DNA samples are analyzed by PFGE at 14 °C in gels run with a 0.1–1.0-s switch time for 16 h at 6 V/cm at an angle of 120° or with a 3.0–30.0-s switch time for 16 h at 6 V/cm depending on the concentration of agarose used.
10. Following PFGE, place the agarose gel in a sealable container containing 400 mL 0.1 μg/mL ethidium bromide and gently shake for 30 min at room temperature.
11. Wash the ethidium bromide-stained agarose gel in 400 mL MQ water by gently shaking for 30 min.
12. Visualize DNA bands using a suitable UV system for analyzing agarose gels. An example of recombinant vaccinia virus DNA digested with the restriction enzyme *Sal I* and analyzed by PFGE is shown in Fig. 6.

3.10 Preparation of rFPV-T7 Stock

Infectious recombinant IBVs are generated in situ by co-transfection of vaccinia virus DNA, containing the modified IBV cDNA, and pCi-Nuc (a plasmid containing the IBV N gene) into CK cells previously infected with a recombinant fowlpox virus expressing the bacteriophage T7 DNA-dependant RNA polymerase under the direction of the vaccinia virus P7.5 early/late promoter 8 (rFPV-T7). This protocol covers the procedure for preparing a stock of rFPV/T7 by infecting primary avian chicken embryo fibroblasts (CEFs).

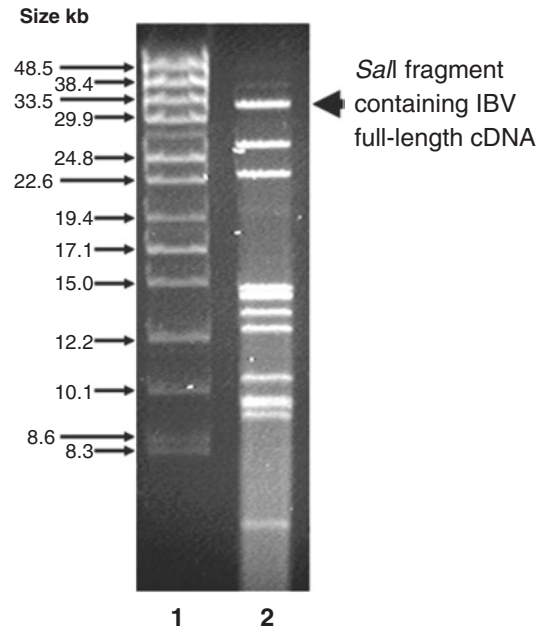

Fig. 6 Analysis of Sal I-digested vaccinia virus DNA by PFGE. Lane 1 shows DNA markers and Lane 2 the digested vaccinia virus DNA. The IBV cDNA used does not contain a Sal I restriction site; therefore the largest DNA fragment (~31 kb) generated from the recombinant vaccinia virus DNA represents the IBV cDNA with some vaccinia virus-derived DNA at both ends

Preparation of a 200 mL stock of rFPV-T7 uses ten T150 flasks containing confluent monolayers of CEFs.

1. Remove the culture growth medium from the cells and infect with 2 mL rFPV/T7 at an MOI of 0.1, previously diluted in CEF maintenance medium.
2. Incubate the infected cells for 1 h at 37 °C 5% CO_2 and then without removing the inoculum add 20 mL of CEF maintenance medium.
3. After 4 days of infection check for CPE (90% of the cells should show CPE). Tap the flasks to detach the cells from the plastic and disperse the cells into the medium by pipetting them up and down.
4. Harvest into 50 mL Falcon tubes and freeze-thaw the cells three times as described in Subheading 3.1, **step 1**.
5. Centrifuge at 750 × *g*, 4 °C for 5 min to remove the cell debris. Take the supernatant containing the virus stock and store at −70 °C until required.
6. Determine the titer of the virus stock using CEF cells. The titer should be in the order of 10^6–10^7 PFU/mL.

3.11 Infection and Transfection of CK Cells for the Recovery of rIBV

1. Wash 40% confluent CK cells in 6-well plates once with PBSa.
2. Infect the cells with rFPV-T7 at an MOI of 10 in 1 mL of CK cell culture medium. Typically we carry out ten replicates per recovery experiment.
3. Incubate for 1 h at 37 °C 5% CO_2.
4. During this infection period prepare the transfection reaction solutions.
5. Solution A: 1.5 mL OPTIMEM, 10 μg rVV DNA, and 5 μg pCi-Nuc per replicate.
6. Solution B: 1.5 mL OPTIMEM and 30 μL lipofectin per replicate.
7. Incubate solutions A and B at room temperature for 30 min.
8. Mix solutions A and B together producing solution AB, and incubate for a further 15 min at room temperature.
9. Remove the rFPV-T7 from each well, wash the CK cells twice with OPTIMEM, and carefully add 3 mL of solution AB per well.
10. Incubate the transfected cells at 37 °C 5% CO_2 for 16–24 h.
11. Remove the transfection medium from each well, replace with 5 mL of BES medium, and incubate at 37 °C 5% CO_2.
12. Two days after changing the transfection media, when FPV/IBV-induced CPE is extensive, harvest the cell supernatant from each well and using a 5 mL syringe, filter through 0.22 μm to remove any rFPV-T7 virus present.
13. Store the filtered supernatant, referred to as passage 0 (P_0 CKC) supernatant at −70 °C.

3.12 Serial Passage of rIBVs in CK Cells

To check for the presence of any recovered rIBVs the P_0 CKC supernatant is passaged three times, P_1–P_3, in CK cells (Fig. 4b). At each passage the cells are checked for any IBV-associated CPE and for further confirmation RNA is extracted from P_3 CKC supernatant and is analyzed by RT-PCR (*see* **Note 12**).

For passage 1 (P_1):

1. Wash the confluent CK cells in 6-well plates once with PBSa.
2. Add 1 mL of the P_0 CKC supernatant per well and incubate at 37 °C 5% CO_2 for 1 h.
3. Without removing the inoculum add 2 mL of BES medium per well.
4. Check cells for IBV-associated CPE over the next 2–3 days using a bright-field microscope.
5. Harvest the supernatant from each well and store at −70 °C.
6. Repeat **steps 1–6** for passages P_2 and P_3 in CK cells.
7. At P_3 any recovered virus is used to prepare a large stock for analysis of the virus genotype and phenotype.

4 Notes

1. Vaccinia virus is classified as a category 2 human pathogen and its use is therefore subject to local regulations and rules that have to be followed.
2. Always discard any medium of solution containing vaccinia virus into a 1% solution of Virkon; leave at least 12 h before discarding.
3. Flasks of cells infected with vaccinia virus should be kept in large plastic boxes, which should be labeled with the word vaccinia and biohazard tape. A paper towel should be put on the bottom of the boxes to absorb any possible spillages.
4. During centrifugation of vaccinia virus-infected cells use sealed buckets for the centrifugation to avoid possible spillages.
5. After 2 h of incubation with the transfection mixture, the cells begin to die. It is best therefore not to exceed 90-min incubation.
6. It is important that after the addition of each selection agent, the medium is mixed to ensure that the selection agents are evenly distributed. This can be achieved by gently rocking/swirling the plate.
7. Add an equal volume of 2% agar to the 2× EMEM containing MPA, xanthine, and hypoxanthine and mix well before adding it to vaccinia virus-infected cells. There is skill to making the overlay medium and adding it to the cells before the agar sets. There are a number of methods including adding hot agar to cold medium, or pre-warming the medium to 37 °C and adding agar that has been incubated at 50 °C. Despite the method chosen it is important that all components of the overlay medium are mixed well, and the medium is not too hot when it is added to the cells. If there are problems, 1% agar can be substituted with 1% low-melting agarose.
8. The first recombinant event in the TDS system will not necessarily occur in the same place in every rVV. It is therefore important to pick a number of plaques from the first round of plaque purification in the presence of GPT selection agents and take a variety of them forward. The following two rounds of plaque purification in the presence of GPT selection agents ensure a single virus population and also that no carry through of the input receiver/wild-type vaccinia virus has occurred.
9. Previous chapters and protocols have instructed during plaque purification in the absence of GPT selection agents to plate 10^{-1} rVV dilution in the presence of GPT selection medium and rVV dilutions 10^{-2} and 10^{-3} in the absence. When there are no plaques in the 10^{-1} dilution, it means that the rVV has

lost the GPT gene and the plaques are ready to amplify and check for the presence of mutations.

10. There are risks associated with working with phenol/chloroform/isoamyl alcohol and chloroform. It is important to check the local COSHH guidelines and code of practices.
11. Vaccinia virus DNA is a very large molecule that is very easy to shear; therefore when working with the DNA be gentle and use wide-bore tips or cut the ends off ordinary pipette tips. In addition always store vaccinia virus DNA at 4 °C; do not freeze as this leads to degradation. However, there is an exception to this if the vaccinia virus DNA has been extracted using the Qiagen QlAamp DNA mini kit, as this DNA will have already been sheared (the kit only purifies intact DNA fragments up to 50 bp). This DNA can be stored at −20 °C but it is only suitable for analysis of the rVV genome by PCR and is not suitable for the infection and transfection of CK cells for the recovery of rIBV.
12. There is always the possibility that the recovered rIBV is not cytopathic. In this case, check for the presence of viral RNA by RT-PCR at passage 3 (P_3). It is quite common even with a cytopathic rIBV not to see easily definable IBV-induced CPE at P_1 and P_2. The recovery process is a low-probability event and the serial passage of rIBVs in CK cells acts as an amplification step.

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Chapter 7

Rescue of Sendai Virus from Cloned cDNA

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Abstract

Sendai virus (SeV) is a non-segment negative-sense RNA virus that naturally infects and causes pneumonia in mice. As a prototypic member of the family *Paramyxoviridae*, SeV has been characterized well, and these studies revealed numerous traits of paramyxovirus biology. The reverse genetics system to rescue SeV was first established in 1995. The virus was rescued from a cloned cDNA that contains full genome sequence flanked by T7 promoter and hepatitis delta virus ribozyme. To rescue SeV, it is necessary to infect cells with a recombinant vaccinia virus vTF7.3 that expresses T7 RNA polymerase, and transfect with the SeV full genome cDNAs together with supporting plasmids encoding NP, P, and L genes under the T7 promoter. Synthesized viral RNA by T7 RNA polymerase will be encapsidated with NP and associated with a polymerase complex composed of P and L. The polymerase complex transcribes and replicates the genome, and produces progeny virions. Rescued SeV needs to be plaque purified to exclude vTF7.3 from viral stock. Reverse genetics system of SeV is relatively efficient compared to other paramyxoviruses, but alternative approaches to rescue poorly growing mutant viruses are also available.

Key words Sendai virus, Rescue, Parainfluenza, Vaccinia virus

1 Introduction

SeV genome is composed of 15,384 nucleotides in size. The virus genome encodes six major structural proteins: nucleocapsid (NP), phospho (P), matrix (M), fusion (F), hemagglutinin–neuraminidase (HN), and large (L) protein. Flanking the viral gene is the putative regulatory 3′ leader (55 nt) and 5′ trailer sequences (57 nt) responsible for directing genomic transcription and replication. In infected cells, both negative-sense viral genome and positive-sense anti-genome exist in the form of a helical ribonucleoprotein (RNP), in which the RNAs are encapsidated by NP protein and associated with the polymerase complex composed of P and L proteins. The L protein is responsible for the majority of enzymatic activities involved in viral replication and transcription. The L protein has a molecular weight greater than 200 kDa and is the least abundant of the structural proteins. The precise composition of the P–L polymerase complex is unclear, but this complex

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can make mRNAs in vitro that is both capped at its 5′ end and polyadenylated at the 3′ end. Expression of the encoded viral proteins relies on the sequential transcription of individual mRNAs by viral polymerase. Transcription of each gene begins with the 10-nt-long transcription initiation signal and ends with the 11-nt-long transcription termination signal. Each gene is also separated by short intergenic sequence composed of 3 nt. The NP protein is predicted to be associated with precisely six nucleotides [1] and paramyxovirus genomes are replicated efficiently only when they are a multiple of six nucleotides in length, a feature known as "the rule of six" [2]. This is an important rule when designing template cDNAs to rescue modified viruses. The modified virus genome should contain exact number of nucleotides of a multiple of six for successful rescue and stable maintenance of the modified genome in the virus.

To rescue SeV from a cloned cDNA, it is necessary to transfect cells with a full-genome cDNA together with supplementary plasmids which express NP, P, and L proteins [3, 4]. These cells also need to be infected with a recombinant vaccinia virus vTF7.3 that expresses T7 RNA polymerase for the expression of the supporting proteins and synthesis of the full-genome viral RNA. The template viral RNA should include the exact 5′ and 3′ terminal sequences to be recognized and replicated by viral polymerase. The critical end of the viral RNA can be generated by self-cleaving ribozyme inserted into the full-genome cDNA. In infected and transfected cells, the viral genome will be encapsidated with NP, and viral polymerase further replicates the genome and transcribes viral mRNAs. Subsequently, all the viral proteins are synthesized and progeny virions are produced. The rescued SeV can be amplified in embryonated eggs and then applied to plaque purification to completely remove the helper virus.

The reverse genetics system for SeV is highly efficient compared to other paramyxoviruses. However, alternative approaches might be required to rescue mutant viruses which replicate poorly in cells. Possible modifications include the use of MVA-T7, a host range-restricted vaccinia virus that expresses T7 RNA polymerase [5]. Interference with SeV replication is less severe with MVA-T7, and MVA-T7 can be eliminated efficiently without the need for any inhibitors by serial passages of the virus in Vero cells [6]. Also, full-genome cDNA template could be modified to include longer and more active form of HDV ribozyme that was shown to significantly improve the rescue efficiency of rabies virus [7]. It is also possible to increase the synthesis of viral RNA by T7 RNA polymerase through inserting three G residues after T7 promoter. In this case, hammerhead ribozymes can be inserted between the promoter and viral gene, which results in the generation of the template RNA possessing the correct 5′-end sequence [7].

2 Materials

2.1 Media

1. Cell culture medium (DMEM-FCS10): Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMax-I, 10% fetal calf serum (FCS), and gentamicin at 50 μg/mL.
2. Culture medium for vTF7.3 (DMED-FCS2): DMEM supplemented with GlutaMax-I, 2% FCS, and gentamicin at 50 μg/mL.
3. Culture medium for SeV (DMEM-BSA): DMEM supplemented with GlutaMax-I, bovine serum albumin fraction V at final concentration of 0.15% (BSA), and gentamicin at 50 μg/mL.
4. Culture medium to amplify SeV (DMEM-BSA-trypsin): For the multistep growth of SeV in cultured cells, add TPCK-treated trypsin at 2 μg/mL to DMEM-BSA.
5. Medium for transfection (OptiMEM): OptiMEM + GlutaMax-I (Gibco).
6. Overlay medium for plaque purification: To prepare 100 mL of overlay medium, make 50 mL of 2× DMEM and 50 mL of 1.8% agarose in different bottles. To make 2× DMEM, add 10 mL of 10× DMEM, 3 mL of 7.5% $NaHCO_3$ solution, 4 mL of 7.5% BSA, 1 mL of GlutaMax-I, 0.1 mL of gentamicin, and 32 mL of sterile water. Then, add 0.5 mL of TPCK-trypsin (1 mg/mL in PBS) (*see* **Note 1**), and keep the medium at 37 °C. To make 1.8% agarose, add 50 mL of H_2O to 0.9 g of agarose, and autoclave the bottle. Keep the bottle in 46 °C water bath after autoclaving. Mix the 2× DMEM (37 °C) and 1.8% agarose (46 °C) just before applying to the cells.

2.2 Cells

1. 293T cells (ATCC, CRL-3216) for the transfection of cDNAs are cultured in DMEM-FCS10.
2. HeLa $T4^+$ cells [8] for the growth of recombinant vaccinia virus vTF7.3 are cultured in DMEM-FCS10.
3. LLC-MK2 (ATCC, CCL-7) for the growth of Sendai virus are cultured in DMEM-FCS10.
4. BS-C-1 cells (ATCC, CCL-26) for plaque titration of vTF7.3 are cultured in DMEM-FCS10.
5. Ten-day-old specific pathogen-free embryonated chicken eggs (Charles River).

2.3 Virus

1. Recombinant vaccinia virus vTF7.3 [9]: The virus expresses T7 phage polymerase, which initiates RNA synthesis under T7 promoter (*see* **Note 2**).

2.4 cDNAs

1. pSeV(E): A plasmid encoding full-genome of SeV strain Enders. The SeV genome in this clone was straddled by a T7 promoter and a hepatitis delta virus ribozyme sequence. The

construction of the plasmid was described previously [10] (*see* **Note 3**).

2. pTF1-SVNP, pTF1-SVP, and pTF1-SVL: Supporting plasmids expressing SeV NP, P, and L proteins. These viral cDNAs were inserted into the pTF1 vector to express the proteins by vaccinia-T7 expression system [11]. The construction of the plasmids was described previously [12, 13].

2.5 Transfection Reagents

1. Lipofectamine 2000 (Invitrogen).

3 Methods

1. To prepare the master stock virus of vTF7.3, infect HeLa $T4^+$ cells in 100 mm dish with the virus at an MOI of one with 1 mL of DMEM-FCS2 and incubate for 2 h at 37 °C and 5% CO_2. Rock the dish by hand at 30-min intervals. Aspirate the inoculum, replace the medium with 10 mL of DMEM-FCS2, and culture for 3 days at 37 °C and 5% CO_2.
2. To harvest the virus, scrape cells together with the medium, transfer to tubes, centrifuge for 5 min at 2000 × *g*, and discard the supernatant. Resuspend the cells in 1 mL of the same medium by gently pipetting or vortexing. Freeze-thaw three times using dry ice/ethanol and 37 °C water bath. Vortex the material vigorously, divide it into cryogenic vials, and store at −70 °C.
3. Prior to infection, add equal volume of trypsin at 0.25 mg/mL and vortex vigorously. Incubate for 30 min at 37 °C, vortexing at 10-min intervals throughout the incubation.
4. To titrate stock virus by plaque assay, prepare BS-C-1 cells in a 6-well plate. Make tenfold serial dilutions of the trypsinized stock virus in DMED-FCS2 and inoculate 1 mL of the diluted virus to each well. Incubate for 2 h at 37 °C in a CO_2 incubator. Remove inoculate, overlay cells with 2 mL of semisolid DMEM-FCS2 containing 0.4% (w/v) carboxy-methyl cellulose, and incubate for 2 days at 37 °C. Remove medium and add 0.5 mL of 0.1% crystal violet to each well. Incubate for 5 min at room temperature (RT), aspirate crystal violet, and allow wells to dry to count the plaque numbers.
5. To rescue SeV, prepare 293T cells in a 6-well plate in DMEM-FCS10. Cells should be passaged to be ~80% confluent at the time of transfection. To obtain the maximal transfection efficiency, prepare the cells a day before transfection and culture in a CO_2 incubator at 37 °C.
6. Infect cells with a UV-inactivated recombinant vaccinia virus vTF7.3 that expresses T7 RNA polymerase (*see* **Note 4**).

Wash 293T cells in 6-well plate with PBS(+) once, add vTF7.3 in 1 mL of DMEM-FCS2 at an MOI of three, and incubate the cells at 37 °C for 2 h.

7. During the incubation, prepare the cDNAs mixed with transfection reagents. To a polystyrene round-bottom tube containing 100 μL of OptiMEM, add 1 μg of pSeV(E), pTF1-SVNP, pTF1-SVP, and 0.1 μg of pTF1-SVL. To the cDNA mixture, add 100 μL of OptiMEM containing 6 μL of Lipofectamine 2000. Mix gently and leave the cocktail at RT for 20 min. Wash cells with PBS(+) three times, and add 1 mL of OptiMEM to the cells. Then, add cDNA-Lipofectamine cocktail to the cells dropwise, gently mix the medium, and culture the cells at 37 °C for 4 h.
8. Remove the inoculate and replace with 1 mL of DMEM-BSA containing 40 μg/mL of cytosine-β-D-arabinofuranoside (AraC, Sigma), which blocks vaccinia DNA synthesis [14]. Culture the cells for 72 h at 37 °C in a CO_2 incubator.
9. After incubation, scrape cells together with the medium and transfer to the centrifuge tube. Lyse the cells by freeze-thawing three times using dry ice and water bath maintained at 37 °C, and inject into 10-day-old embryonated chicken eggs (*see* **Note 5**). Incubate the eggs at 37 °C for 3 days, and then leave the eggs at 4 °C overnight.
10. Harvest the allantoic fluids and centrifuge at 2000 × *g* for 10 min at 4 °C. Test the presence of SeV by standard hemagglutination assay. This passage in eggs increases the population of SeV in the samples, since SeV grows much better than vTF7.3 in embryonated eggs. This process makes it easier for plaque-cloning process that removes vTF7.3.
11. The SeV can be purified by plaque cloning in LLC-MK_2 cells. One or two days before plaque cloning, prepare LLC-MK2 cells in 6-well plate to be confluent on the day of cloning. Make a tenfold dilution of the harvested virus using PBS(+). Depending on the amount of recovered virus, dilution up to $1{:}10^{9–10}$ will be required.
12. Wash cells with PBS(+) three times, add 100 μL of the diluted virus, and leave the plate at RT for 1 h. Occasionally, rock the plate to avoid cells from drying. Remove inoculates and add 2 mL of overlay medium.
13. After overlay medium solidified, place the plate in CO_2 incubator upside down, and culture at 37 °C for 4–5 days.
14. Second overlay can be done to visualize the plaques clearly. The overlay medium can be made as the same way for the first overlay, but add 0.4 mL of 1% neutral red in distilled water to 50 mL of 2× DMEM. Add 1 mL of second overlay medium to

each well, and after solidification, return the plate to the incubator for an extra day.

15. Identify the clear and isolated plaques and take the plaques using Pasteur pipettes. Suspend the cells with 1 mL of PBS(+), and then inoculate to pre-washed LLC-MK2 cells in 6-well plate. Leave the cells at RT for 1 h, remove inoculate, and add 2 mL of DMEM-BSA-trypsin. Culture the cells in the incubator at 37 °C.
16. Check the cells using microscope for the cytopathic effect (CPE). When most of the cells are dead, harvest the supernatant, briefly centrifuge to remove cell debris, and save the supernatant. Rescued SeV clones can be further grown in LLC-MK2 cells or in embryonated eggs to prepare large volume of stock virus.

4 Notes

1. Depending on the cell condition, the amount of trypsin should be adjusted. Here, we usually use trypsin at the final concentration of 5 μg/mL in overlay medium. If strong CPE is observed, the concentration of the trypsin should be reduced.
2. Recombinant vaccinia viruses expressing T7 RNA polymerase have been widely used to rescue SeV and other paramyxoviruses. In addition to the vTF7.3, MVA-T7 can be used to rescue SeV [5]. MVA-T7 is a recombinant vaccinia virus strain Ankara expressing T7 RNA polymerase [15]. This virus does not produce progeny virus in infected mammalian cells. Interference with SeV replication is less severe with MVA-T7, and MVA-T7 can be eliminated efficiently without the need for any inhibitors by serial passages of the virus in Vero cells [6].
3. The pSeV(E) vector is designed so that positive-sense viral RNA is produced by T7 RNA polymerase. The recovery of SeV from a plasmid that produces negative-sense viral RNA genome would be inefficient because co-expressed viral mRNAs expressed from supporting NP, P, and L plasmids would hybridize the viral negative-sense RNA prior to encapsidation with NP [3]. The pSeV(E) vector is over 18 kb in size and should be grown in *E. coli* DH10B to avoid truncation of the cDNA during amplification. The viral gene can be modified to include additional foreign gene of interest. A previous study indicates that up to 3.2 kb can be inserted without reducing viral titer significantly [16].
4. Use of psoralen-treated long-wave UV-irradiated vTF7.3 reduces CPE and significantly improves the efficiency of virus rescue [17]. This treatment inactivated the replication capabil-

ity of the viruses without impairing their infectivity and T7 RNA polymerase expression [18]. To prepare the inactivated virus, add psoralen (49-aminomethyl-trioxsalen; Calbiochem) stock solution (1 mg/mL in H_2O) to the virus (1×10^8 to 1×10^9 PFU/mL) at a final concentration of 0.5 μg/mL. Incubate the suspension, usually 1 mL in a 35-mm-diameter well, at RT for 10 min and irradiate for 10 min in a Stratalinker 1800 UV cross-linking unit (Stratagene) equipped with five 365 nm long-wave UV bulbs.

5. SeV rescued from the transfected 293T cells is not infectious due to the lack of trypsin that activates F protein. However, the culture can be directly injected into the embryonated eggs for growth, because the allantoic fluid contains proteases that cleave and activate the F protein.

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Chapter 8

BAC-Based Recovery of Recombinant Respiratory Syncytial Virus (RSV)

Christopher C. Stobart, Anne L. Hotard, Jia Meng, and Martin L. Moore

Abstract

Respiratory syncytial virus (RSV) is an enveloped, nonsegmented negative-strand RNA virus, which causes lower respiratory tract infections and is a leading cause of mortality in young infants. There is no available RSV vaccine and currently administered prophylactic antibodies are limited to high-risk populations. Current efforts to develop vaccines include development of live-attenuated RSV candidates. We describe here methods for preparation and recovery of recombinant RSV using an efficient bacterial artificial chromosome (BAC)-based system, expansion and plaque purification of recovered virus, and generation of master and working stocks.

Key words Respiratory syncytial virus, RSV, Reverse genetics, Bacterial artificial chromosome, BAC, Virus recovery

1 Introduction

Respiratory syncytial virus (RSV) is an enveloped, negative-strand RNA virus [1, 2]. Since its identification in 1955, RSV has been recognized as a major human respiratory pathogen and remains the leading cause of infant mortality by a virus worldwide [3, 4]. The RSV genome is approximately 15 kb in length and is comprised of ten genes which collectively encode for 11 distinct proteins [2]. The viral replication complex includes the viral proteins: RNA-dependent RNA polymerase (L), phosphoprotein (P), nucleoprotein (N), and a transcription processivity factor, matrix protein 2-1 (M2-1). The first RSV reverse genetics platform for RSV rescue was developed in 1995 [5]. This RSV system involves the co-transfection of a plasmid containing the antigenomic cDNA of RSV strain A2 and four helper plasmids encoding the L, P, N, and M2-1 genes of RSV. Yet, virus rescue systems based on plasmids have demonstrated limited efficiency due to the genetic instability of the cDNA constructs for cloning and recovery [6–8].

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To overcome these limitations, we developed a bacterial artificial chromosome (BAC)-based reverse genetics platform for recombinant RSV recovery [9].

Bacterial artificial chromosomes (BACs) are single-copy plasmid vectors [10]. Cloned DNA instability can cause unwanted expression in bacteria due to cryptic bacterial promoters within the DNA construct. Therefore, difficult-to-clone sequences can be tamed by lowering bacterial growth temperature and/or aeration to reduce bacterial metabolism and by reducing the copy number of the plasmid vector. As single-copy vectors, BACs offer high genetic stability of difficult-to-clone DNA and are additionally capable of modification through recombination-mediated cloning [9, 11]. We describe here the methods of how our RSV BAC reverse genetics platform is employed for recovery of RSV A2-line19F (RSV strain A2 expressing the fusion protein of strain Line 19). This reverse genetics platform consists of a BAC containing the antigenome of RSV A2-line19F under the control of a T7 promoter and four helper plasmids encoding the genes for L, N, P, and M2-1 proteins under the control of a T7 promoter (Fig. 1). We describe below the methods of how the BAC constructs, once acquired, are transformed and propagated in bacteria, the recovery of RSV infectious clones, plaque purification of acquired virus, and generation of high-titer master and working stocks.

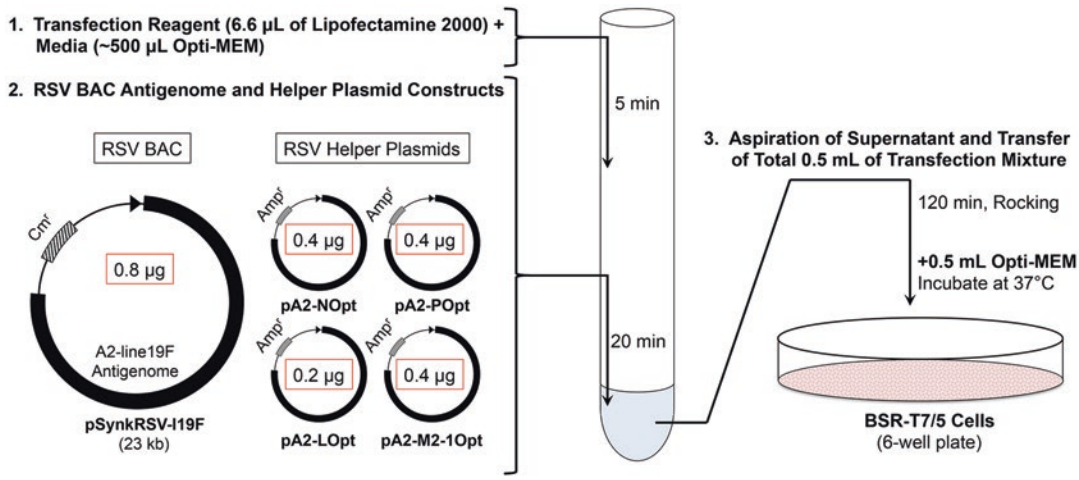

Fig. 1 Overview of the recombinant RSV recovery using the BAC reverse genetics system developed by Hotard et al. [9]. BHK-21 cells expressing T7 polymerase and BSR T7/5 [12] are transfected with BAC construct containing the antigenome of RSV strain A2-line19F under control of a T7 promoter (pro) along with four helper plasmids containing human codon bias-optimized sequences for RSV A2 N, L, P, and M2-1 proteins

2 Materials

All reagents should be stored at 4 °C unless otherwise noted.

2.1 Transformation and Expansion of BAC Constructs

1. Competent high-efficiency *E. coli* 10-β cells (stored at −20 °C until use) and SOC Outgrowth Media.
2. Miller's Luria Broth (LB), prepared by suspending 25 g of LB powder in 1 L of water and autoclaving.

2.2 RSV Recovery Components

1. BHK-21 cells expressing the T7 RNA polymerase gene (clone BSR T7/5) were provided by Dr. Ursula Buchholz [12].
2. 1× Phosphate-buffered saline (PBS), pH 7.2, without calcium and magnesium.
3. Glasgow's MEM (GMEM) supplemented with either 3 or 10% fetal bovine serum and 1% antibiotic–antimycotic solution, which contains 10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B.
4. Lipofectamine 2000 Transfection Reagent.
5. Opti-MEM I, Reduced Serum Medium.
6. RSV BAC Antigenome, pSynkRSV-l19F (may be accessed via BEI Resources/ATCC [NR-36460]) (*see* **Note 1**) [9]. This RSV expresses the far-red fluorescent protein monomeric Katushka-2 (mKate2) in the first gene position [6].
7. RSV Helper Plasmids harboring human codon bias-optimized sequences for L, N, P, and M2-1 proteins: pA2-Lopt [NR-36461], pA2-Nopt [NR-36462], pA2-Popt [NR-36463], and pA2-M2-1opt [NR-36464] (may be accessed via BEI Resources/ATCC) (*see* **Note 2**).

2.3 Plaque Purification and Virus Stock Generation Components

1. HEp-2 cells (ATCC CCL-23) were used for plaque purification.
2. Minimum essential medium (MEM) with Earle's salts (E-MEM) and L-glutamine supplemented with 10% FBS and 1% antibiotic–antimycotic solution which contains 10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B.
3. Overlays were prepared by combining equal volumes of minimum essential medium (MEM) with Earle's salts (E-MEM) and L-glutamine supplemented with 20% FBS and 1% antibiotic–antimycotic solution with 2% agarose.

3 Methods

All procedures should be performed at room temperature unless otherwise noted.

3.1 Transformation and Preparation of RSV BAC Constructs for Transfection

1. 100 ng of the RSV antigenomic BAC were added to a thawed tube of 10-β competent *E. coli* cells on ice. The bacteria were transformed according to the manufacturer's instructions (NEB). In brief, the following steps were performed:
   (a) The bacteria and BAC construct were combined and incubated on ice for 30 min.
   (b) After 30 min, the tube was incubated in a 42 °C water bath to heat shock for 30 s before being returned to ice for 5 min.
   (c) 950 μL of room-temperature SOC media was then added and the mixture shaken at 250 rpm for 1 h at 32 °C.
   (d) Following the 1-h incubation, approximately 100 μL of the bacterial suspension was spread on a pre-warmed (32 °C) agar plate containing 12.5 μg/mL of the selection agent, chloramphenicol.
   (e) The plates were incubated at 32 °C overnight (*see* **Note 1**).
2. After 24 h post-transformation, small well-isolated colonies should be visible (*see* **Note 3**). One to five isolated colonies were used to inoculate an equivalent number of cultures containing 5 mL of LB broth and 12.5 μg/mL of chloramphenicol in 14 mL polypropylene round-bottom tubes.
3. The bacterial cultures were shaken at 32 °C and 250 rpm overnight.
4. Following 24 h of growth at 32 °C, the 5 mL cultures should appear turbid. The small culture(s) may then be used either as a starter culture to inoculate directly a larger culture (a) or via DNA minipreps to analyze constructs (b). Approximately 800 ng of the BAC construct will be required for each recovery transfection (*see* **Note 4**).
   (a) Production of a large culture: The 5 mL culture may be added directly to 200 mL of LB broth containing 12.5 μg/mL of chloramphenicol. The larger culture is shaken at 32 °C and 250 rpm overnight. Following growth, a 0.5 mL aliquot of the bacterial supernatant can be mixed with glycerol (1:1 with 50% glycerol or in a final concentration of approximately 20–25% glycerol) and stored frozen at −80 °C for long-term storage. Purification of the BAC should be performed using a BAC or large-size DNA purification kit.

(b) Isolation of DNA by Miniprep: A small aliquot (~5 μL) of the culture suspension may be plated on a pre-warmed (32 °C) agar plate containing 12.5 μg/mL of chloramphenicol as a selection agent for use as a master plate. Alternatively, an aliquot may be saved as a glycerol stock using the suspension approach described previously. The bacteria are pelleted by centrifugation at 2000×*g* for 10 min at room temperature. Following precipitation of the bacterial culture, the DNA may be isolated using a miniprep protocol. Special care should be taken to follow miniprep protocols (including all steps), which have been established for recovery of low-copy plasmids, or BAC constructs. BAC constructs should be eluted in nuclease-free water. Restriction digests and sequencing may be performed to validate the size and orientation of BAC components prior to inoculating a large culture (*see* **Note 4**).

3.2 Recombinant RSV A2-line19F Recovery

The following protocol describes the methods for transfection of the RSV antigenomic A2-line19F BAC and helper plasmid constructs first described by Hotard et al. [9]. Variations in the recovery time and kinetics of CPE progression may be observed depending upon any changes or modifications made to the RSV genome.

3.2.1 Day −1: Preparation of BSR-T7/5 Cells for Transfection

1. A confluent 60 cm^2 dish of a BSR-T7/5 cell [12] was removed from 37 °C 5% CO_2 incubation and the media aspirated.
2. The cells were washed 1× with a 10 mL of 1× PBS. The wash was aspirated and 1 mL of 0.05% trypsin solution was added to dissociate the cells.
3. Following 3–5 min, the cell monolayer will be visibly dissociated. 26 mL of Glasgow's MEM (GMEM) media supplemented with 10% FBS, 1% penicillin-streptomycin sulfate, and amphotericin B solution was added and the cells were thoroughly mixed to yield a solution of approximately 27 mL of cells.
4. 1 mL of the cell mixture was added to each of the six wells in a six-well plate. An additional 1 mL of 10% FBS GMEM was added to each well and the plate placed in a 37 °C in 5% CO_2 to expand overnight.

3.2.2 Day 0: Transfection of the RSV Antigenomic BAC and Helper Plasmids

Prior to use of the RSV BAC and helper plasmids, all plasmid concentrations should be determined immediately prior to use (*see* **Note 5**). In addition to the BAC antigenome of the virus mutant to be recovered, additional controls are encouraged including the use of a minigenome construct to validate functional helper plasmids, a BAC with an L gene frameshift mutation/nonfunctional L as a negative control for rescue, and a wild-type (A2-line19F) BAC as a positive control for rescue.

1. Prior to transfection, the BSR-T7/5 cells were evaluated for health and confluency. The cells should be at least 95% confluent at the time of transfection (*see* **Note 6**).
2. Transfection mixture calculations were performed to determine the appropriate volumes of the BAC and helper plasmids based on their respective masses to yield a total volume of 500 μL per well. Generally, two replicate wells are transfected for each virus recovery. A 3:1 ratio of Lipofectamine 2000 (μL) to DNA (μg) was used to include the following components: RSV Antigenomic BAC (0.8 μg), Nucleocapsid (N) Helper Plasmid (0.4 μg), Phosphoprotein (P) Helper Plasmid (0.4 μg), M2-1 Helper Plasmid (0.4 μg), Large polymerase (L) Helper Plasmid (0.2 μg), Lipofectamine 2000 Reagent (6.6 μL), and Opti-MEM Reagent (up to 500 μL).
3. Following determination of the reaction volumes to be used, 6.6 μL of Lipofectamine 2000 Reagent was added to the volume of Opti-MEM determined in the previous step and the resulting mixture incubated for 5 min at room temperature.
4. After 5 min, the RSV antigenomic BAC and helper plasmids were added to the mixture and the resulting mixture was incubated for 20 min at room temperature to form transfection complexes. Following these additions, the total volume should be 500 μL for each well to be transfected.
5. After 20 min, the GMEM media was aspirated from each well of the six-well plate of BSR-T7/5 cells and 500 μL transfection complex mixture was added.
6. The plate was rocked slowly at room temperature for 2 h.
7. Following the 2-h incubation, an additional 500 μL of Opti-MEM was added to each well (resulting in a final volume of 1 mL) and the plate was placed in a 37 °C incubator under 5% CO_2 overnight.

3.2.3 Day 1: Replacement of Transfection Inoculum with Media

1. Early the next day (approximately 16 h post-transfection), the transfection inoculums were aspirated from each well (*see* **Note 7**).
2. The wells were washed 1× with a 1 mL of 1× PBS to remove residual transfection complex solution.
3. Following the wash, 2 mL of 3% GMEM (Glasgow's MEM media containing 3% FBS and a 1% penicillin, streptomycin sulfate, and amphotericin B solution) was added to each well.
4. The plate was incubated in a 37 °C, 5% CO_2, incubator overnight.

3.2.4 Day 2: Transfer of Transfected Cells to a Recovery Flask

On day 2, transfected cells that have received the transfected constructs should express mKate2 protein (emission wavelength of 633 nm) and be visible under a far-red channel such as Texas Red (Fig. 2) [13]. However, it will remain difficult to identify

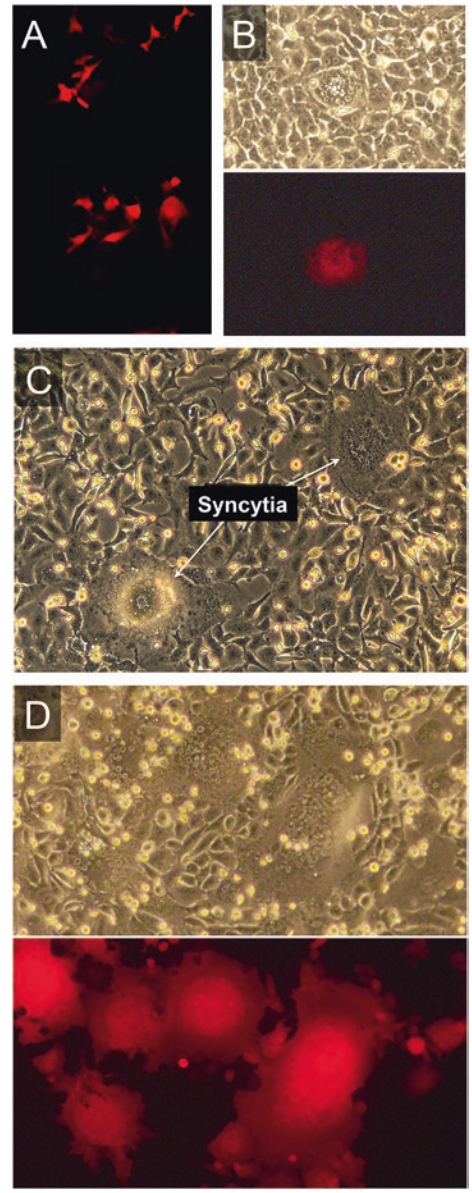

Fig. 2 Images of transfected BSR-T7/5 cells. (**a**) Expression of monomeric Katushka (mKate2) under Texas Red filter channel at 48 h post-transfection. (**b**) An early syncytium in bright-field (*top*) and Texas Red filter channel (*bottom*) at 96 h post-infection. (**c**) CPE evident at 72 h post-transfection. The syncytium on the right remains flattened whereas the syncytium on the left has begun to round up. (**d**) CPE evident at 6 days post-transfection in bright-field (*top*) and Texas Red filter channel (*bottom*)

virus-infected cells by bright field at this time (*see* **Note 8**). The cell density of the BSR cells in the six-well plate should now appear over-confluent and requires passage into a dish or flask.

1. The media from each well was aspirated and the wells washed 1× with 1 mL of sterile 1× PBS.
2. 0.5 mL of 0.05% trypsin solution was added to each well and the plate returned to the incubator for approximately 2 min to facilitate dissociation of the cells. The plate was lightly tapped on each side and the cell monolayer should be visibly detaching from the plate.
3. 3 mL of 3% GMEM was added to the dissociated cells. The resulting mixture was thoroughly mixed and added in its entirety to a T25 (25 cm^2) flask.
4. An additional 1.5 mL of 3% GMEM was added to the flask to result in a total volume of approximately 5 mL.
5. The plate was incubated in a 37 °C, 5% CO_2, incubator overnight.

3.2.5 Days 3–6: Passage of Transfected Cells for Recovery

1. The flasks were monitored for cytopathic effects (CPE) over subsequent days (*see* **Note 9**). When the cells reached 100% confluency, they should be passaged into a subsequent T25 (25 cm^2) flask at a 1:3 split ratio (*see* **Note 10**).
2. Depending on the RSV mutant's growth characteristics, the virus infection will progress from small syncytia to larger syncytia and to rounded syncytial masses (*see* **Note 9**, Fig. 2).
3. Once the monolayer appears to be involved in greater than 50% syncytial masses, the virus may be harvested. Note: Use of the L frameshift control will result in red cells due to L provided by the helper plasmids; however, these cells will not progress to virus-derived syncytia.

3.2.6 Day 7: Harvest of Virus BSR Stock

1. At the time of harvesting, CPE should be evident throughout the flask with at least half of the monolayer involved in syncytial masses (Fig. 2d). Approximately five cryovial tubes should be labeled for harvesting of the virus stock.
2. The monolayer is dissociated into the media with a cell scraper (*see* **Note 11**). The cell suspension is transferred to a 15 mL conical tube and the mixture vortexed for approximately 15 s.
3. Once vortexed, the virus-cell mixture is transferred in 1 mL aliquots into the labeled cryovials and placed in storage at −80 °C for plaque purification and/or master stock generation.

3.3 Plaque Purification of RSV

Plaque purification (optional) can be performed. Once plaques have been picked, they may be stored for future use at −80 °C.

3.3.1 Day –1: Preparation of Cells for Plaque Purification

Six-Well plates of HEp-2 cells should be split for each virus plaque purification to be performed. At the time of infection, HEp-2 cells should be approximately 70% confluent. Described below are the directions for how a six-well plate may be prepared from a confluent 60 cm^2 dish of HEp-2 cells.

1. A confluent 60 cm^2 dish of a HEp-2 cells was removed from incubation at 37 °C and the supernatant aspirated.
2. The cells were washed 1× with a 10 mL of 1× PBS to remove residual media. And 1 mL of 0.05% trypsin solution was applied to dissociate the cells.
3. After approximately 3–5 min, the monolayer of cells will be visibly dissociated. 25 mL of E-MEM (MEM containing Earle's salts and L-glutamine, Gibco) media containing 10% FBS and a 1% penicillin, streptomycin sulfate, and amphotericin B solution (Invitrogen) was added and the cells were thoroughly mixed to yield a solution of approximately 26 mL of cells.
4. 1 mL of the cell mixture was added to each of the six wells in a six-well plate. An additional 1 mL of E-MEM was added to each well and the plate placed in a 37 °C under 5% CO_2 to expand approximately threefold overnight.

3.3.2 Day 0: Infection for Plaque Purification

1. To serially dilute, a 96-well plate was prepared by adding 270 μL of cold MEM (lacking FBS and supplements) to each of the rows (with the exception of the first row) of a 96-well flat-bottom plate, which was kept on ice.
2. 300 μL Neat BSR-derived stocks was placed in the first empty row.
3. 30 μL of the neat virus stock was then transferred to the next row representing a 1:10 dilution. The mixture was pipetted up and down ten times to ensure homogeneity. The process was repeated until dilutions were completed through the $1:10^{-7}$ dilution.
4. Following virus serial dilution, the six-well plates were checked for ~70% confluency before the media supernatants are removed by aspiration.
5. 200 μL of each of the virus supernatant for dilutions 10^{-2} to 10^{-7} is applied to wells of the plate. Following inoculation, the plate was rocked slowly at room temperature for 1 h to adsorb.
6. Following 1-h incubation, 2 mL of a 50:50 suspension of 20% FBS EMEM and 2% agarose were applied as an overlay to each of the wells (*see* **Note 12**). At the time of addition, the suspension should feel warm to touch (~40–45 °C).
7. After approximately 5–10 min at room temperature, the plates are placed in a 37 °C, 5% CO_2, incubator.

3.3.3 Day 1 to Day ~5: Visible Plaque Formation

Over the next 3–5 days, plaques/fluorescent foci should appear. Every other day, 1 mL of the 50:50 suspension of 20% EMEM and 2% agarose should be added to each well to help sustain cell viability. The time required to form visible plaques will vary considerably based on the specific virus phenotype.

3.3.4 Day 6: Plaque Isolation

Around day 6, visible plaques, which are well isolated, should be present in one of the dilutions in the plate.

1. Holding the plate up to the light, potential visible plaques are circled with a fine-tipped marker. The circled plaques are examined under a microscope to verify that the circle contains the plaque near center.
2. Using a 1 mL serological pipette, the opening of the pipette is placed directly over the circled area and firmly pressed down until it reaches the base of the well (*see* **Note 13**). The pipette is then used to scrape the well plastic to dislodge the cells and overlay plug. Once dislodged, the scrapings are drawn into the serological pipette and transferred into a 1.5 mL microcentrifuge tube containing 1 mL of E-MEM. As RSV is largely cell associated, it is important to scrape the cells with the tip of the serological prior to pipetting.
3. The process is repeated for the acquisition of at least five plaques.
4. The tubes are briefly vortexed before being stored at −80 °C or directly used.

3.4 Generation of Master/Working Stocks

Normally, a master stock is recovered, split into aliquots, and an aliquot is used for generation of a working stock. Master stocks represent the first stock of virus generated either directly from the BSR-derived virus stock or from plaque. When working with plaques, not all plaques result in productive master stock infections. Several attempts at recovering a master stock from plaque stocks may be required. RSV stocks may be grown in several different cell lines including Vero or HEp-2 cells. Note, however, that RSV can decline in virus titer over time at −80 °C and requires occasional re-titration of infectivity and regeneration of working stocks.

3.4.1 Day −1: Preparation of Cells for Virus Recovery

On the day before infection for master or working stock generation, HEp-2 (or other, e.g., Vero) cells should be passaged to yield a T182 (182 cm^2) flask at approximately 70–80% confluency next day.

3.4.2 Day 0: Infection for Virus Recovery

Prior to infection, the plaque to be used for master or working stock generation should be thawed at 37 °C temperature. Ideally, the flask will be ready for infection near the time of thawing completion.

1. Supernatant media in the T182 (182 cm^2) flask is aspirated and 2 mL of serum-free MEM is added to the flask.
2. Approximately 1 mL of the plaque suspension is added to the flask yielding a mixture of 3 mL of virus suspension for infection.
3. The flask is incubated at room temperature while rocking (as described above).
4. After 1 h, an additional 47 mL of pre-warmed 10% E-MEM is added and the flask is placed in a 37 °C incubator under 5% CO_2. Note that some mutants may propagate better at 32 °C [14].
5. The virus will typically take approximately 4–6 days to reach the extent of infection optimal for harvesting the master stock.

3.4.3 Day ~5: Harvest of Virus Stock

At 5 days post-infection, the cell monolayer should be at least 50% involved in syncytia or show signs of infection, as evidenced by mKate2 fluorescence. Common signs of late-stage infection include extensive cytoplasmic effects such as adherent multinucleated syncytia and large rounded syncytia that have begun the process of detaching from the flask surface. Some strains induce less syncytia than others but spread in HEp-2 monolayers nonetheless, and therefore the mKate2 signal is informative for time of harvest. The flask is ready for harvesting when most of the monolayer exhibits signs of infection and the monolayer may have begun to detach from the flask.

1. Once the flask has reached the point of harvesting, the cells are scraped into the media and are split between two 50 mL conical tubes.
2. The tubes of cells are vortexed for approximately 15 s and chilled on ice for 5 min.
3. Following ice incubation, the tubes are sonicated on ice at 30% amplitude for pulses of 1 s with a pause of 1 s between each. The number of pulses is equivalent to the number of mL of cells in the tube.
4. Following sonication, the tubes are centrifuged for 10 min at $2000 \times g$ at 4 °C to pellet residual cell debris.
5. The supernatant from the tubes is pooled into a fresh 50 mL conical on ice and is aliquoted at approximately 1 mL per tube into cryogenic tubes.
6. The aliquots are then rapidly frozen by submersion into either liquid nitrogen or a bath of dry ice and isopropanol. The virus aliquots should be stored at −80 °C and re-titrated over long storage due to slow degradation of virus.

4 Notes

1. The RSV BAC includes the strain RSV A2 expressing the fusion (F) protein of Line 19. Previous studies have shown that the F protein of Line 19 is associated with higher lung viral load, lung IL-13 levels, increased mucin expression, and higher airway dysfunction in mice compared to strains A2 and Long F. The BAC contains selection resistance for chloramphenicol. Generally, the BAC is grown in bacteria at 32 °C rather than 37 °C for increased genetic stability while cloning and culturing.
2. The BAC and helper plasmids may be accessed through BEI Resources/ATCC as described. The helper plasmids are codon optimized for expression and are under T7 promoter control. The details for design of these plasmids are described in Hotard et al. [9].
3. The colonies observed following transformation of the BAC might vary in size.
4. There are many different restriction enzymes, which may be used to develop a restriction fingerprint of the BAC. EcoRV digestion of the pSynkRSV-l19F BAC results in seven fragments (7.5, 5.6, 3.3, 3.2, 2.7, 0.2, 0.2 kb).
5. Depending upon the DNA isolation kit used for large-scale purification, many kits involve a terminal DNA precipitation. If this approach is used, it is imperative that the RSV BAC is homogenously dissolved in solution prior to concentration determination and transfection. Due to the nature of the experimental approach used, inaccuracies in DNA concentrations may greatly reduce virus recovery efficiency and yield.
6. The confluency of the monolayer must be high at the time of transfection with Lipofectamine. Transfections can be performed with lower levels (<95%) of cell confluency; however lower transfection efficiencies and thus viral recoveries may occur.
7. The next day the cells will not likely show any apparent signs of virus infection. However, if the virus possesses the monomeric Katushka (mKate) protein, dim red cells should be visible after 24-h infection indicating successful transfection.
8. At 48 h post-transfection, cells under bright field may start to form rounded syncytia exhibiting 2–8 nuclei. Many times it is difficult to know for sure, however, the mKate2 under red channel should exhibit a strong signature if these masses are true syncytia.
9. Between 3 and 6 days post-transfection, active syncytia should begin to grow and the virus infection should become apparent. The monolayer should go through phases beginning with small syncytia, then larger syncytia, then syncytia that begin to round up, and lastly detachment from the monolayer (Fig. 2).

10. Since RSV is predominately cell associated, very little concern should be made for retaining or recycling media when passaging.
11. It is important that the entire monolayer is scraped into the overlying supernatant to optimize a strong BSR-derived stock for generating master stocks.
12. The temperature of the plaque overlay must be carefully monitored prior to use. If the temperature is too hot (the media is too hot to touch), the cells will be scorched and the monolayer will be lost. If the temperature is too cold (very little or no detectable warmth against the skin), the suspension will harden before there is time for addition. Care should be taken when adding to add the overlay to the side of the wells rather than directly into the well and not too fast as such actions may detach the monolayer.
13. To ensure disruption of the cells associated with a plaque and that the plaque has been detached, the surface must be adequately disrupted before aspirating the plaque to ensure uptake of the virus and plaque.

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Chapter 9

Recovery of a Paramyxovirus, the Human Metapneumovirus, from Cloned cDNA

B.G. van den Hoogen and R.A.M. Fouchier

Abstract

Human metapneumovirus (HMPV), a single-stranded negative-sense RNA virus belonging to the family *Paramyxoviridae*, is associated with respiratory tract illness, primarily in young children and persons with underlying disease. Based on genetic and antigenic variation, HMPV strains are classified into two serotypes, with isolates NL/1/00 and NL/1/99 as prototypes for serotypes A and B, respectively. The development of plasmid-based reverse genetics systems for both serotypes has resulted in developments of a wide range of vaccine candidates against HMPV infection. The approach to virus rescue of HMPV is similar to that used for other paramyxoviruses, starting with mini-replicon assays for optimizations of the rescue protocols and subsequent replacement of the mini genome with a plasmid expressing the cDNA of the full-length viral RNA genome. Here, we provide detailed information on the reverse genetics systems for HMPV.

Key words Human metapneumovirus, HMPV, Single-stranded negative RNA virus, Reverse genetics, Virus rescue

1 Introduction

Human metapneumovirus (HMPV), a paramyxovirus identified in 2001, is a leading cause of respiratory tract infections (RTIs) in both children and adults [1]. HMPV virions contain a lipid membrane envelope containing the three surface glycoproteins, the fusion (F), attachment (G), and small hydrophobic (SH) proteins. On the basis of sequence diversity between the fusion (F) and attachment (G) protein genes and the difference in virus neutralization titers, two main HMPV lineages A and B were identified, each divided into two genetic sublineages: A1 (prototype virus strain NL/1/00), A2, B1 (prototype virus strain NL/1/99), and B2 [2]. Inside the envelope lies a helical ribonucleocapsid (RNP) complex, which consists of the nucleoprotein (N), the polymerase cofactor phosphoprotein (P), the viral RNA polymerase (large protein, L), and the non-segmented single-stranded negative-sense RNA genome. The HMPV genomic RNA is approximately 13 kb

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in length and consists of at least eight genes that potentially encode at least nine proteins. In contrast to other paramyxoviruses, members of the *Pneumovirinae* subfamily express the M2 gene, encoding two partially overlapping open reading frames (ORFs), which give rise to two proteins M2-1 and M2-2. The M2-1 protein is a transcription anti-termination factor that is important for the efficient synthesis of full-length mRNAs as well as for the synthesis of poly-cistronic read-through mRNAs. The M2-2 protein is thought to direct the switch between virus RNA replication and transcription. Expression of M2-2 protein was found not to be necessary for successful recovery of recombinant respiratory syncytial virus (RSV) with reverse genetic techniques [3].

HMPV infects cells upon binding of the attachment protein to the cellular receptor on the host cells, followed by fusion of the viral and cellular membranes directed by the viral F protein (Fig. 1). Upon entry of the cells, the viral RNP containing the negative-sense genome is released into the cytoplasm, where virus replication takes place. At early times of infection, the viral genome serves as template for the synthesis of viral messenger RNA (mRNA) and the negative-sense genome is replicated after translation of these transcripts and accumulation of the viral proteins. Replication results in a full-length complementary copy, the positive-sense antigenome, which serves as a template to direct the synthesis of the genomic RNA. This genomic RNA contains short extragenic regions at the 3′ and 5′ends, referred to as the leader and trailer regions. The 3′ end of the viral RNA (vRNA) directs both replication and transcription, while the 5′ end of the genome contains signals that direct the replication of the antigenome. The genome of HMPV further contains noncoding regions between each gene that range in size from 23 to 209 nucleotides and contain gene end signals, intergenic regions, and gene start signals [4]. For RSV and other paramyxoviruses it is known that these gene end and gene start sequences control transcription termination and re-initiation, leading to a gradient of mRNA abundance that decreases from the 3′ end of the genome (N gene) toward the 5′ end (L gene) [5]. After transcription and translation, the viral envelope proteins F, G, and SH are transported through the endoplasmic reticulum (ER) and the Golgi apparatus to be inserted in the plasma membrane. During a budding process at the site of the plasma membrane, the viral components such as the RNP, glycoproteins, and M protein are assembled to form progeny viruses.

We have established a reverse genetic system to recover infectious virus of two prototypes (NL/1/00 and NL/1/99) of HMPV [6], which has been used in several studies [7–10]. Here, we provide detailed information on the reverse genetics systems for HMPV. For successful recovery of negative-sense RNA viruses such as HMPV, precise 5′ and 3′ ends of the genomes are required for replication and packaging of the genomic RNA, and the viral RNA

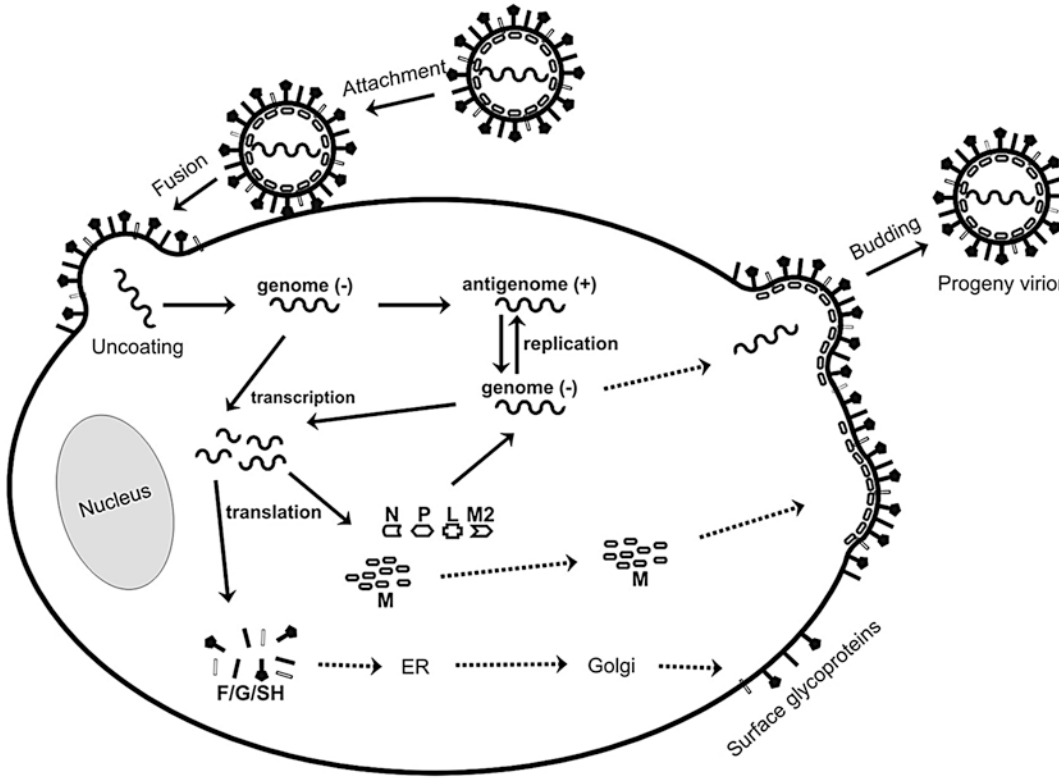

Fig. 1 Schematic representation of the HMPV replication cycle. Upon attachment of the virion to the plasma membrane and subsequent fusion of the viral and plasma membranes, the virion is uncoated and the RNP (containing the negative-sense genome) is released in the cytoplasm. Here, the negative-sense RNA genome serves as a template to produce mRNAs that can be translated into proteins and to produce a positive-sense copy of the RNA genome (antigenome). The antigenome is used as a template to synthesize genomic RNA. Upon genome transcription and replication and after translation, M proteins and RNPs are transported intracellularly (*see dotted lines*) to the plasma membrane and the viral glycoproteins F, G, and SH from the ER to Golgi to plasma membrane. At last, assembled virions are released from the plasma membrane by a budding process

polymerase is essential for transcription to produce messenger RNA (mRNA) and RNA replication (negative sense to positive and vice versa). The reverse genetics system for HMPV comprises the use of a plasmid containing the cDNA encoding the full-length viral RNA in the positive-sense orientation under the control of a T7 RNA polymerase promoter-terminator cassette. Upon transcription by T7 RNA polymerase, an exact 3′ terminus of the initial transcript of the HMPV cDNA is generated by autolytic cleavage by a hepatitis delta virus (HDV) ribozyme, which is positioned at the 3′ end of the genome. The exact 5′ end of the transcript is determined by positioning the site of the T7 promoter as close to the terminus of the initial transcript as possible resulting in transcription by T7 RNA polymerase immediately adjacent to the T7 polymerase promoter sequence (Fig. 2). To increase transcription from the T7

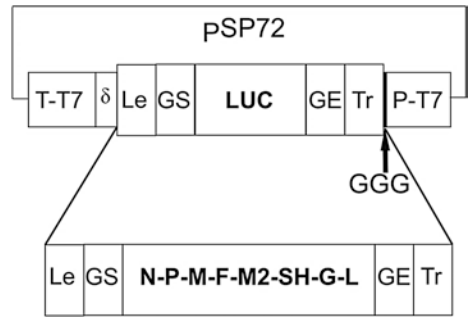

Fig. 2 Schematic representation of the mini-replicon construct. The firefly-luciferase (LUC) reporter gene is flanked by the viral leader sequence (Le) and gene start signal (GS) of the nucleocapsid gene on the *left hand*, and the gene end signal (GE) of the polymerase gene and the viral trailer (Tr) on the other end. This mini-replicon was cloned in the context of a T7 RNA polymerase promoter-terminator cassette (P-T7, T-T7). The authenticities of the transcribed ends of the negative-sense mini-replicon are determined by the position of the T7 promoter sequence and an HDV ribozyme sequence (δ). To increase transcription from the T7 promoter, three G residues were placed between the T7 promoter and the end of the trailer sequence. The full-genome cDNA plasmid used to produce recombinant HMPV is the same as the mini-replicon construct, but with the Le-GS-LUC-GE-Tr cassette replaced by a full cDNA copy of the HMPV genome (*bottom panel*)

promoter, three G residues were placed between the T7 promoter and the end of the trailer sequence [11]. The positive-sense antigenomic RNAs cannot be directly used for the translation of proteins, since it has terminal sequences that extend beyond those in the mRNAs, and therefore cannot be translated efficiently. Expression plasmids for the N, P, L, and M2-1 proteins, which are also under the control of the T7 promoter, are therefore co-transfected to ensure sufficient production of proteins required for virus RNA transcription and replication. Mini-replicon and full-length cDNA constructs are genotype specific and therefore the expression plasmids for N, P, M2-1, and L proteins need to contain the sequence of the same virus as where the mini-replicon and full-length construct are derived from.

The T7 RNA polymerase can be expressed either from an expression plasmid or by cells constitutively expressing the T7 RNA polymerase, the BSR-T7 cells (baby hamster kidney cells stably expressing T7 polymerase) [12, 13]. Before using plasmids encoding the full-length viral genome, mini-replicon systems can be used to test the fidelity of the sequences of the genomic termini, test whether the plasmids encoding the viral polymerase complex components are functional, and optimize rescue conditions. Mini-replicon systems consist of a reporter gene, such as luciferase (LUC), chloramphenicol acetyl transferase (CAT), or fluorescent proteins, flanked by the viral leader and GS of N at one side and

the GE of L and trailer at the other side, which are enclosed by a T7 promoter/terminator cassette and a self-cleaving ribozyme, as described above. Co-transfection of the expression plasmids for L, N, P, and M2-1 proteins also under the control of T7 promoters, with the mini-replicon construct, will result in expression of the reporter gene for which expression levels can be quantified. Upon optimization, optimal conditions can be used for virus rescue using the mini-replicon system in which the reporter gene is replaced by the full-length viral genome. Mini-replicon and rescue assays are described for both 293-T cells (with co-transfection of an expression plasmid for T7 polymerase) and BSR-T7 cells (expressing T7 polymerase). Where mini-replicon assays are usually more efficient on 293-T cells, BSR-T7 cells are more efficient for virus rescue.

2 Materials

2.1 Solutions and Kits

1. 10× Stock of 2× HBS: 8.18% NaCl, 5.94% HEPES, and 0.2% Na_2HPO_4 (all w/v). Filter-sterilize and store at 4 °C. Make a 2× solution with H_2O and adjust the pH to 7.12 with 1 M NaOH. Store in aliquots at −20 °C (*see* **Note 1**).
2. Dual-Glo® Luciferase Assay system.
3. CAT Elisa kit (Roche).
4. Transfection reagent Lipofectamine 2000.
5. Iscove's modified Dulbecco's medium (IMDM).
6. Dulbecco's modified Eagle medium (DMEM).
7. Opti-MEM® medium.

2.2 Cells and Media

Pre-warm all media to room temperature prior to handling of the cells. Incubate cells in an incubator at 37 °C, 5% CO_2. Heat-inactivate fetal calf serum (FCS) for 30 min at 56 °C, aliquot, and store at −20 °C.

1. Vero cells (ATCC® CCL-81™) grown in culture medium: IMDM supplemented with 10% FCS, 100 IU of penicillin/mL, 100 µg of streptomycin/mL, and 2 mM glutamine. Passage cells once a week at a 1:20 to 1:40 dilution.
2. Cocultivation medium for Vero cells: IMDM supplemented with 3% FCS, 100 IU penicillin/mL, 100 µg streptomycin/mL, 2 mM glutamine, and 1 µL/1 mL 2.5% trypsin solution (2.5 µg/mL) (*see* **Note 2**).
3. Infection medium for Vero cells: IMDM supplemented with 100 IU penicillin/mL, 100 µg streptomycin/mL, 2 mM glutamine, and 15 µL/100 mL 2.5% trypsin solution (0.375 mg/mL).

4. 293-T cells (ATCC® CRL-3216™) grown in culture medium: Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FCS, 1 mM sodium pyruvate, 1× nonessential amino acids (100× for MEM Eagle), 100 IU penicillin/mL, 100 μg streptomycin/mL, and 2 mM glutamine. Passage cells twice a week at a 1:10 to 1:40 dilution. Supplement with 0.5 mg of G418/mL (*see* **Notes 3** and **4**).
5. 293-T transfection medium: DMEM, supplemented with 1× nonessential amino acids (100× for MEM Eagle), 1 mM sodium pyruvate, 100 IU penicillin/mL, 100 μg streptomycin/mL, 2 mM glutamine, 3% FCS, and 1 μL/1 mL 2.5% trypsin solution (2.5 μg/mL).
6. BSR-T7 cells (obtained from K. Conzelmann, Munich, Germany) grown in culture medium: DMEM, supplemented with 1× nonessential amino acids (100× for MEM Eagle), 1 mM sodium pyruvate, 100 IU penicillin/mL, 100 μg streptomycin/mL, 2 mM glutamine, and 10% FCS. Passage cells twice a week at a 1:10 to 1:40 dilution. Supplement with 0.5 mg G418/mL (*see* **Note 4**).
7. BSR-T7 rescue medium: DMEM, supplemented with 1× nonessential amino acids (100× for MEM Eagle), 1 mM sodium pyruvate, 100 IU penicillin/mL, 100 μg streptomycin/mL, 2 mM glutamine, 3% FCS, and 1 μL/1 mL 2.5% trypsin solution (2.5 μg/mL).

2.3 Plasmids

1. Polymerase complex constructs: The construction of the expression plasmids for the polymerase proteins was described [6]. In brief, the individual ORFs of N, P, M2-1, and L of either NL/1/00 or NL/1/99 were cloned in the multiple cloning site of pCITE to yield plasmids pCITE-N, pCITE-P, pCITE-M2-1, and pCITET-L. The start codons of the ORFs were adapted to the Kozak sequence: ccaATGg. Plasmids should be stored at −20 °C at 1 μg/μL in sterile H_2O.
2. Mini-replicon constructs: The construction of the mini-replicon constructs has been described previously [6]. Briefly the construct consists of plasmid pSP72 (Promega) in which the NdeI (nucleotide [nt] 2379)-to-HpaI (nt 136) fragment is replaced by a synthetic T7 promoter extended with three G residues, two BsbI sites, the HDV ribozyme (δ), and a T7 terminator to yield pSP72-P_{T7}-δ-T_{T7}. A fragment containing the HMPV genomic leader sequence, with the gene start of N, two BsmBI sites, and the genomic trailer sequence with the gene end of L (specific for NL/1/00 or NL/1/99), was placed in pSP72-P_{T7}-δ-T_{T7} to yield pSP72-P_{T7}-Tr-BsmBI-BsmBI-Le-δ-T_{T7}. The ORFs of green fluorescent protein (GFP), CAT, or LUC cloned in the BsmBI sites between the N gene start and L gene end signals

of HMPV yielded pSP72-P_{T7}-Tr-GFP-Le-δ-T_{T7}, pSP72-P_{T7}-Tr-CAT-Le-δ-T_{T7}, or pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7}, respectively. Constructs were stored at −20 °C at a concentration of 1 μg/μL in sterile H_2O.

3. pRL-SV40 Vector (Promega), which constitutively expresses *Renilla* luciferase.

   This plasmid is used as control in mini-replicon assays with pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7}.

4. Expression plasmid for T7 polymerase, such as TargeTron Vector pAR1219 (Sigma-Aldrich), or pAR3126 as described by Dunn et al. [13].

5 Full-length cDNA vectors were based on the mini-replicon plasmids pSP72-P_{T7}-Tr-LUC/CAT/GFP-Le-δ-T_{T7} of HMPV strains NL/1/00 and NL/1/99, in which the reporter genes were replaced by the full-length genomic sequences (*see* [6]).

3 Methods

3.1 Coating 6-Well Plates or 10 cm Dishes with Gelatin for Use with 293-T Cells (See Note 3*)*

1. Warm gelatin to 37 °C and make a 1:20 dilution in sterile PBS (from 2% stock).
2. Add 1 mL per well of a 6-well plate.
3. Incubate for 15 min at room temperature.
4. Aspirate gelatin/PBS and wash with 1 mL PBS.
5. Aspirate PBS, make sure that the wells are dry, and leave at room temperature.

3.2 Mini-Replicon Assays Using pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7} and 293-T Cells

In this assay the Luciferase system is used. The pRL-SV40 Vector plasmid serves as a transfection and sample processing control. One day prior to transfection, coat wells with gelatin (*see* Subheading 3.1), plate 3×10^5 293-T cells per well of a 6-well plate in 2 mL 293-T culture medium, and place plates in the 37 °C incubator overnight.

1. Next day, transfect cells at the end of the afternoon (*see* **Note 5**).
2. Bring 2× HBS and medium to room temperature before transfection.
3. Bring pRL-SV40 Vector plasmid to a concentration of 10 ng/μL and all other plasmids to 0.2 μg/μL. The expression plasmids for N, P, M2-1, and L need to contain the sequences of the same virus as where the mini-replicon construct is based on. For instance, if the mini-replicon construct is based on NL/1/00, the expression plasmids need to code for NL/1/00 proteins.
4. For one transfection reaction (1 well of a 6-well plate) mix the following materials in a 1 mL Eppendorf tube:

Mini-replicon construct pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7} (0.2 μg/μL)	5 μL
pRL-SV40 (10 ng/μL)	5 μL
T7 expression plasmid (0.2 μg/μL)	7.5 μL
pCITE-N (0.2 μg/μL)	4 μL
pCITE-P (0.2 μg/μL)	2 μL
pCITE-M2-1 (0.2 μg/μL)	2 μL
pCITE-L (0.2 μg/μL)	2 μL
2 M $CaCl_2$	6.2 μL
Sterile H_2O	To 50 μL

The expression plasmids for N, P, M2-1, and L need to contain the sequences of the same virus as where the mini-replicon construct is based on. For instance, if the mini-replicon construct is based on NL/1/00, the expression plasmids need to code for NL/1/00 proteins.

5. Dropwise, add 50 μL 2× HBS; do not vortex!
6. Incubate exactly for 5 min at room temperature to allow a fine milky precipitate to develop. Do not vortex or shake! (*See* **Note 6**.)
7. Meanwhile refresh 293-T cells with 2 mL/well 293-T culture medium.
8. After 5 min, briefly blow air bubbles in the tube with a pipet to ensure proper mixture.
9. Carefully add the transfection mix (100 μL/well) dropwise to the cells.
10. Incubate overnight in a 37 °C incubator; the next morning refresh medium with 293-T culture medium.
11. At 48 h after transfection proceed to the Luciferase assays (*see* Subheading 3.4).
12. Mini-replicon assays using pSP72-P_{T7}-Tr-GFP-Le-δ-T_{T7} can be read out by fluorescence microscopy or with a flow cytometer. Mini-replicon assays using pSP72-P_{T7}-Tr-CAT-Le-δ-T_{T7} can be read out by a CAT-ELISA (*see* **Note 7**).

3.3 Mini-Replicon Assays Using pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7} and BSR-T7 Cells

1. One day prior to transfection seed 0.8–1 × 10^6 BSR-T7 cells per well of 6-well plate, to reach 80–95% confluency the next day, and place the 6-well plates in the 37 °C incubator overnight.
2. Next day, bring pRL-SV40 at 10 ng/μL, mini-replicon construct at 0.2 μg/μL, and all other plasmids at 0.1 μg/μL.
3. Bring Opti-MEM medium, BSRT-7 culture medium (without addition of G418), and Lipofectamine at room temperature.

4. Prepare two tubes and add to tube #1:

Opti-MEM medium	250 µL
Mini-replicon construct pSP72-P_{T7}-Tr-LUC-Le-δ-T_{T7} (0.2 µg/µL)	15 µL
pRL-SV40 (10 ng/µL)	5 µL
pCITE-N (0.1 µg/µL)	3 µL
pCITE-P (0.1 µg/µL)	3 µL
pCITE-M2-1 (0.1 µg/µL)	2.5 µL
pCITE-L (0.1 µg/µL)	1.5 µL

The expression plasmids for N, P, M2-1, and L need to contain the sequences of the same virus as where the mini-replicon construct is based on. For instance, if the mini-replicon construct is based on NL/1/00, the expression plasmids need to code for NL/1/00 proteins.

5. Add, for each transfection, in tube #2: 250 µL Opti-MEM medium and 10 µL Lipofectamine.
6. Incubate at room temperature for 4 min.
7. Transfer the 250 µL from tube 2 to tube 1 and gently pipet up and down.
8. Incubate at room temperature for exactly 20 min; longer incubation will reduce transfection efficiency.
9. In the meanwhile, refresh medium (2 mL/well) of cells with BSR-T7 culture medium (without addition of G418).
10. Add transfection mixture to the wells and incubate for 5 h in a 37 °C incubator.
11. Refresh medium with BSR-T7 culture medium (without addition of G418).
12. After 72 h, proceed to the Luciferase assay (*see* Subheading 3.4).

3.4 Luciferase Assay for Mini-Replicon Assay Expressing Luciferase

1. Remove media from cells.
2. Add 1 mL PBS, pipet the cells loose from the surface, and transfer to a 1.5 mL tube.
3. Spin at 400 × *g* for 3 min and aspirate PBS.
4. Resuspend cells in 200 µL PBS.
5. Transfer 75 µL to a black or white 96-well plate (Corning).
6. Add 75 µL Dual-Glo reagent per well.
7. Incubate for 10 min, mix carefully, and avoid air bubbles.
8. Measure Firefly-luciferase chemiluminescence in a plate reader.
9. Make a 1:100 dilution of Stop&Glow substrate in Stop&Glow buffer.

10. Add 50 µL of this solution to the 96-well plate.
11. Measure Renilla-luciferase chemiluminescence in a plate reader (*see* **Note 8**).
12. Calculate Firefly/Renilla ratio (relative light units: RLU) and the fold increase: ratio between RLU sample and RLU-negative sample.
13. Efficient mini-replicon assay normally results in chemiluminescence values (*see* **Note 7**: read out for the pSP72-P_{T7}-Tr-CAT-Le-δ-T_{T7} mini-replicon system).

3.5 Rescue of Recombinant Virus

1. Virus rescue on 293 T cells
   (a) One day prior to transfection, coat 6 cm dishes with gelatin (*see* Subheading 3.1) and seed 3×10^5 293-T cells per well.
   (b) The next day, transfect cells at the end of the afternoon (*see* **Note 5**).
   (c) Bring all plasmids to a concentration of 0.2 µg/µL.
   (d) Pre-warm 2× HBS and medium to room temperature.
   (e) Add per single transfection in a tube (one reaction):

Full-length construct (0.2 µg/µL)	25 µL
T7 expression plasmid (0.2 µg/µL)	7.5 µL
pCITE-N (0.2 µg/µL)	4 µL
pCITE-P (0.2 µg/µL)	2 µL
pCITE-M2-1 (0.2 µg/µL)	2 µL
pCITE-L (0.2 µg/µL)	2 µL
2 M $CaCl_2$	6.2 µL
Sterile H_2O	To 50 µL

   The expression plasmids for N, P, M2-1, and L need to contain the sequences of the same virus as where the full-length construct is based on. For instance, if the full-length construct is based on NL/1/00, the expression plasmids need to code for NL/1/00 proteins.
   (f) Mix briefly and dropwise add 50 µL 2× HBS.
   (g) Incubate exactly for 5 min at room temperature to allow a fine milky precipitate to develop. Do not vortex or shake! (*See* **Note 6**.)
   (h) Meanwhile refresh 293-T medium: 2 mL/well (293-T culture medium).
   (i) After 5 min, briefly blow air bubbles in the tube with a pipet.
   (j) Add the 1 mL transfection mixture dropwise to the cells in the 10 cm dish.

(k) Incubate overnight in a 37 °C incubator. Refresh medium the next morning with 293-T transfection medium.

(l) Incubate in a 37 °C incubator for 72 h until proceeding to coculture (*see* Subheading 3.5, **step 3**).

2. Virus rescue on BSR-T7 cells

(a) One day prior to transfection, seed approximately 8×10^6 BSR-T7 cells per well of a 6-well dish, to ensure 80–90% confluency the next day.

(b) Next day, bring full-length construct at 0.2 μg/μL and all other plasmids at 0.1 μg/μL in sterile H_2O.

(c) Bring Opti-MEM, BSRT-7 transfection medium, and Lipofectamine at room temperature.

(d) Add per transfection in tube #1:

Opti-MEM medium	250 μL
Full-length construct (0.2 μg/μL)	15 μL
pCITE-N (0.1 μg/μL)	3 μL
pCITE-P (0.1 μg/μL)	3 μL
pCITE-M2-1 (0.1 μg/μL)	2.5 μL
pCITE-L (0.1 μg/μL)	1.5 μL

The expression plasmids for N, P, M2-1, and L need to contain the sequences of the same virus as where the full-length construct is based on. For instance, if the full-length construct is based on NL/1/00, the expression plasmids need to code for NL/1/00 proteins.

(e) Add per transfection in tube #2: 250 μL Opti-MEM medium and 10 μL Lipofectamine.

(f) Incubate at room temperature for 4 min.

(g) Bring the 250 μL from tube 2 to tube 1 and gently pipet up and down.

(h) Incubate at room temperature for exactly 20 min; longer incubation times will decrease the transfection efficiency.

(i) In the meantime, refresh medium on cells with 2 mL BSRT-7 transfection medium.

(j) Add transfection mixture to the wells and incubate for 5 h in a 37 °C incubator.

(k) Refresh medium with BSRT-7 transfection medium.

(l) Incubate in a 37 °C incubator for 72 h before proceeding to coculture on Vero cells (*see* below).

3. Coculture on Vero cells

(a) One day prior to coculture, seed 1×10^6 Vero cells in T-25 flask (Corning).

(b) Next day, carefully wash transfected 293-T or BSR-T7 cells with PBS.

(c) Aspirate PBS and add 4 mL Vero cocultivation medium.

(d) Scrape the transfected cells loose with a rubber cell scraper and bring cell/medium suspension to a 15 mL tube.

(e) Add 2 mL of this suspension to the T-25 flask with Vero cells.

(f) Incubate in a 37 °C incubator.

(g) After 3 days, and every 3 days, refresh with Vero infection medium.

(h) At appearance of first signs of cytopathic effects (6–10 days), refresh the infection medium, next day scrape cells loose with a rubber cell scraper, and collect medium/cell suspension (*see* **Notes 9–11**).

(i) Use this suspension directly to generate passage 1 (*see* Subheading 3.5, **step 4**) or after a quick freeze-thaw round (−80 °C for at least 4 h, followed by quick thaw at 37 °C) followed by removal of cell debris through centrifugation at 400 × *g*, 5 min (*see* **Note 12**).

(j) Store aliquots by adding 1:1 50% (w/w) sucrose solution and freeze aliquots at −80 °C.

4. Generation of passage 1

(a) One day prior to inoculation, seed 5 × 10^6 Vero cells (in medium containing 10% FCS) in a T-75 flask (Corning).

(b) Next day, wash cells with PBS or medium without FCS.

(c) Add 1 mL of coculture and 4 mL of Vero infection medium and incubate for 2 h in 37 °C incubator.

(d) Add 10 mL of Vero infection medium and incubate flasks in a 37 °C incubator.

(e) Refresh medium every 2 days.

(f) At appearance of first signs of cytopathic effects, replace the infection medium (7–14 days) (*see* **Notes 9–11**), next day scrape cells loose with a rubber cell scraper, and collect cell suspension.

(g) Freeze-thaw this suspension once: −80 °C for at least 4 h, followed by a quick thaw at 37 °C. Remove cell debris through centrifugation at 400× *g*, for 5 min (*see* **Note 11**).

(h) Add 1:1 50% (w/w) sucrose solution to the supernatant and freeze aliquots at −80 °C.

(i) Test for the presence of virus by means of virus titrations following standard protocols.

(j) Generally, virus rescue results in a titer of around 1×10^6 $TCID_{50}$/mL.

4 Notes

1. 10× Stock of 2× HBS: The pH of this solution is of outmost importance to the success of transfection efficiency. Determination of the pH should be conducted with a well-calibrated pH meter. Upon getting the pH at 7.12, the solution should be kept at room temperature and pH must be measured again 1 h later. Only when the pH is stable at two consecutive measurements, the solution can be stored in aliquots at −20 °C. The solution should be at room temperature prior to use in transfection experiments, to assure the proper pH value necessary for transfection.
2. The addition of trypsin to the culture medium for HMPV is necessary for effective cleavage of the fusion protein. However, too high concentrations of trypsin will result in detachment of the cells from the flask. Therefore, the proper concentration of trypsin has to be defined by optimization when other sources of trypsin are going to be used. Optimization is done by testing for the maximum amount of trypsin at which the cells do not detach from the plates. The concentration of trypsin is usually around 0.2–0.4 mg/mL.
3. The 293-T cells are fragile when cultured in 6-well or 10 cm dishes. To prevent detachment from the plates during transfection, the plates should be coated with 0.1% gelatin as described under Subheading 3.1. This coating is only necessary when cells are subsequently used for transfection or infection, and not when cells are passaged.
4. To keep expression of the large T antigen in the 293-T cells and T7 in the BSR-T7 cells, geneticin (G418) is added once a week to the culture medium. Geneticin is not added when cells are plated for subsequent use in transfection or infection experiments.
5. Transfection with the $CaPO_4$ method should be done for a minimum of 7 h, but overnight is usually better. Longer duration of transfection will result in toxicity of the cells. The next morning, the cells should be covered with little speckles (the precipitates).
6. It is important to add the 2× HBS dropwise. While doing so, you should see a milky white precipitation occurring. The incubation of exactly 5 min at room temperature is important to achieve high transfection efficiency.
7. For mini-replicon assays with the CAT reporter gene, 0.4 μg of pTS27, a vector expressing β-galactosidase under the control of a cytomegalovirus immediate-early promoter (gift of Dr. M. Malim) is co-transfected; *see* Herfst et al. [14]. The β-galactosidase functions, like the pRL-SV40, as transfection and sample

processing control. Mini-replicon assays using the CAT expressing system can be read out by enzyme-linked immunosorbent assay for CAT and β-galactosidase (Roche Diagnostics) according to the instructions from the manufacturer and mini-replicons expressing GFP can be read out with a flow cytometer equipped with an argon laser emitting at 488 nm (Becton Dickinson).

8. A plate reader is needed that enables reading chemiluminescence, for instance a Tecan Infinite-200 reader (www.tecan.com/Infinite-200).
9. With all HMPV cultures, medium is refreshed the night before the cultures are harvested. This prevents the accumulation of defective virus particles [15].
10. Cytopathic effects are not always visible for HMPV-infected Vero cells. Blind passage at day 14 might result in cytopathic effects in later passages. Alternatively, immune fluorescence assays or FACS analysis with anti-HMPV antibodies will reveal the presence of infectious virus.
11. HMPV virions are mostly present in cell-associated form, with only a small portion excreted to the supernatant. To harvest viruses from the cells, the supernatant is harvested together with the cells and this suspension subjected to one freeze-thaw cycle. After thawing the cell debris should be removed by centrifugation at 400 × *g*, for 5 min.
12. HMPV is sensitive to heat inactivation. Leaving the virus suspension too long at 37 °C, or room temperature, results in reduction of infectious virus titers. During freeze-thaw cycles, thawing the virus stocks is best conducted at 37 °C with constant examination. The ampules should be placed on ice immediately upon being thawed.

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Chapter 10

Reverse Genetics of Newcastle Disease Virus

Stivalis Cardenas-Garcia and Claudio L. Afonso

Abstract

Reverse genetics allows for the generation of recombinant viruses or vectors used in functional studies, vaccine development, and gene therapy. This technique enables genetic manipulation and cloning of viral genomes, gene mutation through site-directed mutagenesis, along with gene insertion or deletion, among other studies. An in vitro infection-based system including the highly attenuated vaccinia virus Ankara strain expressing the T7 RNA polymerase from bacteriophage T7, with co-transfection of three helper plasmids and a full-length cDNA plasmid, was successfully developed to rescue genetically modified Newcastle disease viruses in 1999. In this chapter, the materials and the methods involved in rescuing Newcastle disease virus (NDV) from cDNA, utilizing site-directed mutagenesis and gene replacement techniques, are described in detail.

Key words Newcastle disease virus, Reverse genetics, Virus rescue, Site-directed mutagenesis, Gene replacement, cDNA, Cell culture

1 Introduction

Reverse genetics has become an essential tool to study viruses and their host interactions. This technique involves the genetic manipulation of viral genomes in order to understand their function and interaction with host cells. It also allows for the generation of recombinant viruses or vectors utilized in vaccine development and gene therapy [1–3].

Reverse genetics has been employed to engineer DNA and RNA viruses. The very first genome manipulations were performed in DNA viruses followed next by RNA viruses [2]. Poliovirus was the first positive-stand RNA virus to be recovered in 1981 [2]. Manipulation of negative-strand RNA virus genomes was complicated by several factors including the requirement of a precise genome length for replication and packaging, the requirement of the RNA polymerase for initial viral replication and mRNA synthesis, the need for a ribonucleoprotein (RNP) complex, and lastly the

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fact that some negative-stranded RNA viruses possess segmented genomes [2].

Overcoming multiple difficulties, the influenza virus was the first negative-strand RNA virus to be successfully manipulated and recovered [4]. However, the task was still challenging for non-segmented negative-strand RNA viruses, until the successful recovery of rabies virus was achieved in 1994, by co-transfecting plasmids encoding the NP, P, and L genes with a plasmid encoding the antigenome of the rabies virus (all of which contained the bacteriophage T7 polymerase promoter) into cells infected with a recombinant vaccinia virus expressing the RNA polymerase from bacteriophage T7 [2, 5]. This system was rapidly adopted for the manipulation and recovery of other non-segmented negative-strand RNA viruses [6–10], including Newcastle disease virus (NDV), rescued for the first time in 1999 by Dr. Peeters and collaborators [11].

The development of reverse genetics for NDV has allowed the genetic manipulation of its genome to achieve a better understanding of viral functions during replication and infection [11–16]. NDV reverse genetics has made possible the development of a valuable recombinant vaccine system, enabling expression of its own mutated proteins or foreign proteins, thus opening opportunities to investigate its applications as recombinant vaccines, as a multivalent vaccine candidate for poultry, and as a vaccine vector for other animal species and humans [14, 17–30]. NDVs modified by reverse genetics have also become valuable candidates for anticancer therapy in humans [31–35].

In this chapter, we focus on the description of a successful technique to recover infectious clones of NDV from a full-length cDNA, a site-directed mutagenesis protocol to attenuate the fusion protein cleavage site, and a method for fusion and hemagglutinin-neuraminidase gene replacement. This virus rescue technique consists of (1) a recombinant modified vaccinia virus Ankara that expresses the RNA polymerase from bacteriophage T7 (MVA/T7) [36]; (2) three helper plasmids containing the bacteriophage T7 polymerase promoter that encode the NP, P, and L genes from NDV [12]; and (3) a full-length cDNA plasmid containing the bacteriophage T7 polymerase promoter and terminator flanking the full-length antigenome of the desired NDV strain or modified NDV.

The MVA/T7 virus was generated from the highly attenuated MVA virus derived with more than 570 passages of the vaccinia virus Ankara strain in chicken embryo fibroblasts. These continuous passages resulted in the loss of MVA replication in mammalian cells [37] by preventing virus assembly [38], but did not affect its ability to express viral and recombinant genes [38]. Thereafter, this MVA was used to generate the MVA/T7 that expresses the RNA polymerase gene from bacteriophage T7 [36]. The MVA/T7 system has been used by several research groups to recover infectious clones of genetically modified NDV for multiple applications [12, 14, 17–19, 39, 40].

Generally, the helper plasmids expressing the NP, P, and L genes from NDV have been developed by different laboratories using a variety of cloning vectors; however, all of these plasmids are similar in function and structure. The helper plasmids referred to in this chapter's protocol were developed by Dr. Yu and collaborators at the Southeast Poultry Research Laboratory [12]. Of note, the full-length cDNA plasmid development procedure may vary between research groups (*see* refs. [12, 18, 40, 41] for further details).

In general, for the use of the cDNA with the MVA/T7 system, a cDNA spanning the full antigenome of the selected NDV strain is generated first by multiple overlapping partial reverse transcriptase PCR (RT-PCR) amplifications, using total RNA extracted from the allantoic fluid of NDV-infected embryonated chicken eggs. Next, the multiple overlapping fragments are sequentially cloned together, either through compatible restriction site ligation or through the use of a cloning kit, into a modified low-copy-number plasmid containing a T7 polymerase promoter, the sequence of the hepatitis delta virus ribozyme (HDV Rz) and the T7 terminator; the multi-cloning site (MCS) is located between the T7 polymerase promoter and the HDV Rz, where the amplified cDNA will be inserted [12, 18, 40, 41]. The HDV Rz will generate precise 3′ ends by auto cleavability and ensure the appropriate size of the viral genome, complying with the rule of six [3, 42] (i.e., genome size must be a multiple of six nucleotides; Fig. 1). The full-length cDNA plasmids can be used as a backbone to delete genes, replace or insert foreign genes into NDV genome to study their function, and/or to create recombinant vaccine vectors [12–15, 19, 25, 28, 39].

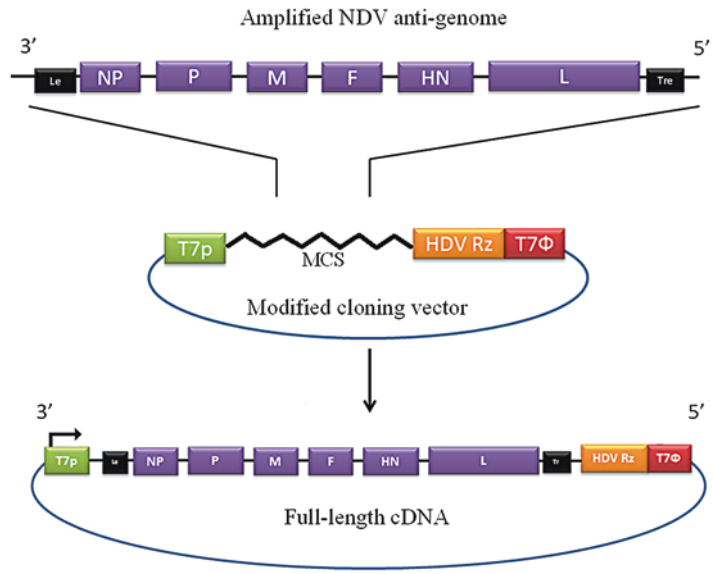

Fig. 1 Construction of a full-length anti-genomic cDNA plasmid

Once the full-length antigenome plasmid has been constructed, viral rescue ensues. As mentioned above, this system requires infection of the mammalian cell line (Hep-2) with MVA/T7, which will express and produce the T7 RNA polymerase. Infected cells will then be co-transfected with the full-length cDNA plasmid and the three helper plasmids. The T7 RNA polymerase will bind the promoters on the helper plasmids and on the full-length cDNA to start transcription and translation for virus replication and assembly.

In the following sections, the reagents, materials, and step-by-step methodology needed for site-directed mutagenesis, fusion gene replacement, and recovery of recombinant NDV are listed in detail. All protocols must be performed under the proper biosafety level and following appropriate biosafety guidelines (*see* to **Note 1**).

2 Materials

2.1 NDV Genome Manipulation

2.1.1 Attenuation of the Fusion Protein Cleavage Site Through Site-Directed Mutagenesis

1. Total RNA extracted from the allantoic fluid of NDV-infected eggs.
2. Fusion gene primer set (forward and reverse, 0.15–0.5 μM each).
3. Mutagenic primer set (forward and reverse, 0.5 μM each), phosphorylated at the 5′ and PAGE purified.
4. Hi-fidelity RT-PCR kit and instructions manual (i.e., SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Invitrogen, cat. #12574-035).
5. Site-directed mutagenesis kit and instructions manual (i.e., Phusion Site-directed mutagenesis kit, Thermo Fisher Scientific, cat. #F-541).
6. DNA gel extraction kit and instructions manual (i.e., QuickClean II Gel Extraction Kit, GenScript, cat. #L00418).
7. Topo pCR2.1 cloning vector (Invitrogen, cat. # K4500-02).
8. Top10 chemically competent *E. coli* with provided S.O.C. media (Invitrogen, cat. # C4040).
9. Plasmid purification kit and instructions manual (i.e., Qiagen plasmid miniprep, cat # 27106).
10. Nuclease-free water.
11. Agarose.
12. Distilled/deionized water.
13. SYBR® Safe gel staining (Invitrogen, cat. #S33102).
14. 1 kb Ladder.
15. Luria Bertani (LB) agar plates supplemented with ampicillin (100 μg/mL).

16. Luria Bertani (LB) broth supplemented with ampicillin (100 μg/mL).
17. X-Gal, 40 mg/mL working solution for blue/white colony screening.
18. Pipettes and pipette tips.
19. 1.5 mL Microcentrifuge tubes.
20. 250 μL PCR tubes.
21. Sterile 15 mL conical or round-bottom culture tubes.
22. Sterile bacterial cell spreaders.
23. Ice.
24. Water bath capable of reaching temperatures of 42 and 55 °C.
25. Thermocycler.
26. DNA gel electrophoresis system.
27. Bacteriology incubator set at 37 °C.
28. Rocking incubator set at 37 °C.

2.1.2 NDV Gene Replacement

1. Full-length cDNA plasmid containing the antigenome of the desired NDV strain (vector).
2. Intermediate plasmid containing the genes to be replaced (insert) into the vector (i.e., F and HN).
3. Gene-specific primer set (forward and reverse) to linearize the vector (10 μM each).
4. Gene-specific cloning primer set for the insert (10 μM each).
5. pfuULTRA™ II Fusion HS DNA polymerase (Stratagene, cat. #600672).
6. In-Fusion® HD Cloning Kit and instructions manual (Clontech, cat. #).
7. DNA gel extraction kit and instructions manual (GenScript).
8. Max Efficiency® Stabl2™ competent *E. coli* (Invitrogen).
9. Plasmid purification kit and instructions manual (Qiagen).
10. Nuclease-free water.
11. Luria Bertani (LB) agar plates with ampicillin (100 μg/mL), or any other antibiotic depending on the resistance gene present in the vector to be used.
12. Ice.
13. Sterile bacterial cell spreaders.
14. Thermocycler.
15. Tabletop micro-tube centrifuge.
16. Bacteriology incubator set at 37 °C.
17. Rocking incubator set at 30 °C.

2.2 Cell Culture Growth and Maintenance

1. High-glucose Dulbecco's modified Eagle medium (DMEM) (1×), liquid (Gibco, cat. # 21068-028).
2. Penicillin (10,000 UI/mL)/streptomycin (10,000 mg/mL) 100× solution, cell culture grade (i.e., Gibco, cat. # 15140-122).
3. Heat-inactivated fetal bovine serum (FBS) (i.e., Gibco, cat. # 16140-071).
4. Phosphate-buffered saline (PBS) (1×) (i.e., Gibco, cat. # 10010).
5. 0.25% Trypsin-EDTA (i.e., Gibco, cat. # 25200).
6. Serological pipettes (5 and 10 mL) and pipettor.
7. T25 and T75 cell culture flasks.
8. Water bath.
9. Centrifuge capable of holding 15 and 50 mL conical tubes.
10. Cell culture incubator set at 37 °C with a 5% CO_2 atmosphere.

2.3 Infection-Transfection

1. High-glucose Dulbecco's modified Eagle medium (DMEM) (1×) liquid (Gibco).
2. Opti-MEM I Reduced-Serum Medium (1×) liquid (i.e., Gibco, cat. # 31985062).
3. Phosphate-buffered saline (PBS) (1×) (Gibco).
4. Penicillin (10,000 UI/mL)/streptomycin (10,000 mg/mL) 100× solution, cell culture grade (Gibco).
5. Heat-inactivated fetal bovine serum (FBS) (Gibco).
6. Lipofectamine® 2000 (Invitrogen).
7. Porcine pancreatic trypsin (i.e., Sigma, cat. # T5266).
8. Full-length cDNA plasmid encoding full NDV genome.
9. Helper plasmids encoding NDV NP, P, and L genes.
10. Recombinant modified vaccinia virus Ankara expressing the T7 RNA polymerase (MVA/T7).
11. Hep-2 cells (human origin, HeLa cell contaminant) (ATCC CCL-23).
12. Non-coated 6-well cell culture plates.
13. Serological pipettes (5 and 10 mL) and pipettor.
14. Single-channel pipettes able to dispense from 1 to 1000 μL.
15. Filtered pipette tips.
16. Sterile microcentrifuge tubes (1.5 mL).
17. Sterile polypropylene conical tubes (15 and 50 mL).
18. Biosafety cabinet class II.
19. Cell culture incubator set at 37 °C with a 5% CO_2 atmosphere.

20. Centrifuge capable of holding 15 and 50 mL conical tubes.
21. Inverted microscope.
22. Hemocytometer or automated cell counter.
23. 2–4% Trypan Blue.

2.4 Propagation of the Rescued Virus in Embryonated Chicken Eggs (ECEs)

1. 9- to 11-day-old specific pathogen-free (SPF) ECEs.
2. Cleared Hep-2 cell culture supernatant containing the rescued virus.
3. Ethanol/iodine mix (70% ethanol/30% iodine).
4. Glue to seal eggs.
5. Egg puncher or a 16G × 1.5″ needle with a rubber stopper.
6. Tuberculin syringes with 25G × 5/8″ needles.
7. 5 mL Syringes with 16G × 1.5″ needles.
8. 15 mL Conical tubes.
9. 2 mL Screw-cap, O-ring tubes or 1.8 mL cryovials.
10. Biosafety cabinet class II.
11. Egg incubator set at 37 °C.

3 Methods

3.1 NDV Genome Manipulation

3.1.1 Attenuation of the Fusion Protein Cleavage Site Through Site-Directed Mutagenesis (Fig. 2)

1. Amplify the fusion gene-coding region in a single fragment, using the extracted RNA as template, a hi-fidelity RT-PCR kit of your choice, and fusion gene-specific primer set.
2. Analyze the amplicons by DNA gel electrophoresis using 0.7–1% agarose gels.
3. Excise the band at approximately 1700 bp.
4. Purify the PCR product using the DNA gel extraction kit of your choice, following the manufacturer's instructions.
5. Clone the purified PCR product into TOPO pCR2.1 vector, following the manufacturer's instructions.
6. Transform the cloning product into TOP10 chemically competent *E. coli*, following the manufacturer's instructions.
7. Pre-warm the LB plates containing 100 μg/mL of ampicillin, spread 40 μL of X-Gal working solution on the surface, and allow air-drying.
8. Plate 100 μL of the transformed *E. coli* suspension on LB plates and incubate overnight at 37 °C (between 16 and 24 h).
9. After incubation blue (plasmid with no insert) and white (plasmid with insert) bacterial colonies will be observed on the LB plates. Pick up between 5 and 15 white colonies with sterile toothpicks or 20 μL pipette tips.

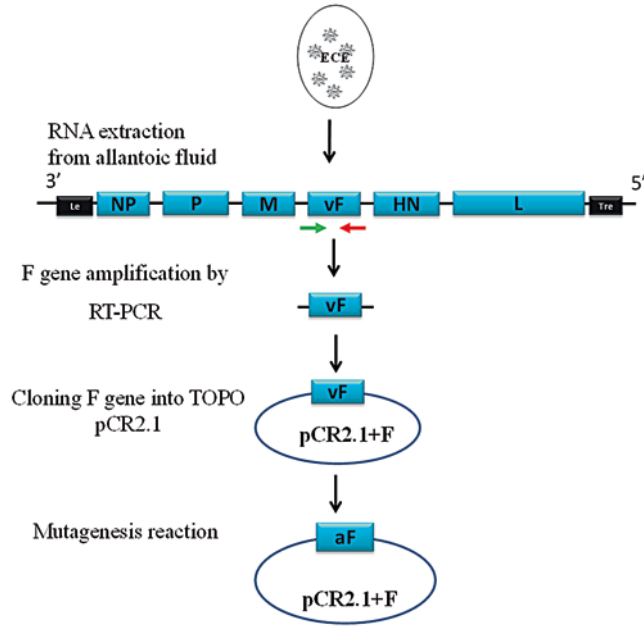

Fig. 2 Amplification, subcloning, and mutation of the F gene

10. Inoculate each colony into separate 15 mL sterile conical tubes or round-bottom culture tubes containing 5 mL of LB broth plus 100 μg/mL of ampicillin.
11. Incubate overnight at 37 °C, 225 rpm. Loosen the tube caps to allow air into the tubes during incubation.
12. After incubation, purify the plasmids using the plasmid purification kit of your choice, following the manufacturer's instructions.
13. The purified plasmids can be screened by size through electrophoretic analysis utilizing the plasmid from a blue colony as a negative control.
14. Confirm plasmids showing the expected size by sequencing analysis.
15. Once sequences have been confirmed proceed to site-directed mutagenesis following the chosen kit's manufacturer's instructions (i.e., Phusion Site-directed Mutagenesis kit, Thermo Fisher Scientific).
    (a) Locate the cleavage site of the fusion protein and identify the nucleotides that need to be mutated in order to convert the virulent cleavage site into an a-virulent cleavage site using the LaSota strain as reference.
    (b) Design a forward primer (mutagenic primer) containing the nucleotide changes, and a reverse primer. Both primers have to be phosphorylated at the 5′ end (to be able to re-circularize the plasmid) and be PAGE purified. The forward primer

mutagenic sequence has to be flanked by 10–15 perfectly matched nucleotides on each side, and the annealing temperatures should fall between 65 and 72 °C (refer to the kit's manual for details on primer design; i.e., forward 5′-ATC TGG AGG G**g**G GAG ACA G**gg** ACG C**c**T TAT AGG TGC CG-3′, reverse 5′-GTG GAC ACG GAC CCT TGT ATC CTA CGG ATA GAA TCG CCC-3′).

(c) Perform the PCR reactions as directed in the mutagenesis kit using the fusion-gene plasmid. This reaction will amplify the full plasmid and yield a linearized product.

(d) Analyze the mutagenesis PCR product through gel electrophoresis, excise the band showing the expected size, and perform DNA gel extraction.

(e) To circularize the plasmid contained in the mutagenesis PCR product, use the T4 DNA ligase provided with the mutagenesis kit and follow the manufacturer's instructions.

(f) Transform circularized plasmid into TOP10 chemically competent cells following **steps 6–13** from this section.

(g) Confirm the sequence of the mutated fusion gene through sequencing analysis (*see* **Note 2**).

3.1.2 Gene Replacement (i.e., the Attenuated Fusion Gene and the Hemagglutinin-neuraminidase (HN) Gene from One NDV Strain into Another NDV Strain; Fig. 3)

1. *Design vector primers.* The vector primers are to exclude the coding region of the fusion and the HN genes. The 5′ end of the forward primer has to start right after the stop codon of the HN gene, and the 5′ end of the reverse primer has to start right before the start codon of the HN gene. These primers do not require special purification process (i.e., forward 5′-CTA GTT GAG ATC CTC AAA GAT GAC GGG-3′; reverse 5′-ATG ATC TGG GTG AGT GGG CGG-3′) (*see* **Note 2**).
2. *Design primers for the insert.* According to the cloning kit manual, the insert primers have to be between 18 and 25 bp in length. These primers require a gene-specific region and a vector-specific region located at the 5′ end of both forward and reverse primers. The vector-specific region requires 15 nucleotides that match the vector at the site where insertion will occur to facilitate cloning of the insert into the plasmid containing the rest of the NDV genome. PAGE purification is suggested for this set of primers (*see* cloning kit manual for detailed instructions on primer design) (i.e., forward 5′-**act cac cca gat cat** CAT GGT ACT GGA TAA TGA TCT ACT TTG ATT GTT CGT-3′; reverse 5′-**gag gat ctc aac tag** CAA AGG ACC GAT TCT GAA CTC CCC GAA TAG-3′) (*see* **Note 2**).
3. Amplify vector and insert through PCR using the previously designed primers (**steps 1** and **2** of this section) and the pfuULTRA™ II Fusion HS DNA polymerase following the manufacturer's instructions (*see* **Note 3**).

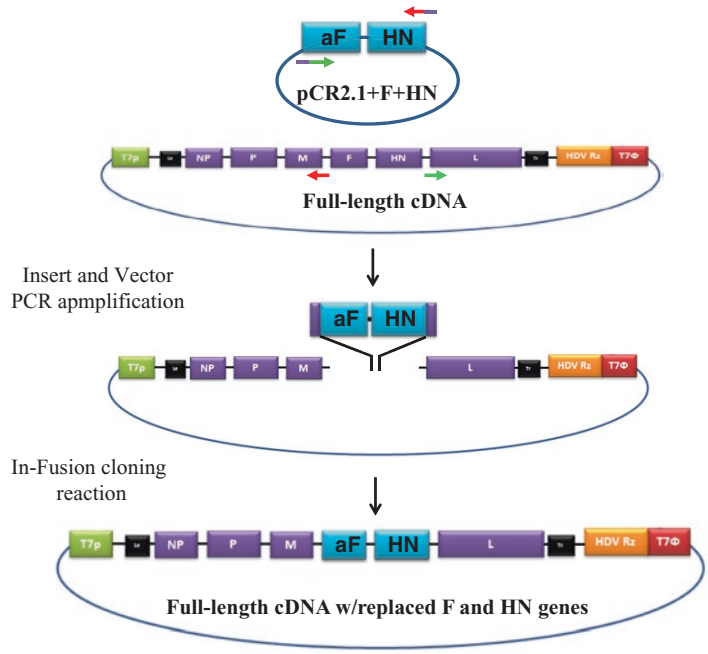

Fig. 3 Gene replacement

4. Analyze the PCR products for both vector and insert through gel electrophoresis, excise the bands showing the appropriate size, and gel purify the products.
5. Quantify the DNA concentrations to ensure that there is enough DNA for the cloning reactions, which will require between 50 and 200 ng of vector and insert, respectively. It may be necessary to prepare more than one PCR reaction for vector amplification in order reach the required DNA concentration (*see* **Note 3**).
6. Prepare the cloning reactions in 10–20 μL final volume (depending on the vector and insert concentrations).
7. Place reactions in a thermocycler, incubate for 15 min at 50 °C, and then place reactions on ice. At this point, the cloning reactions can be stored at −20 °C or can be used to transform competent cells.
8. Proceed to transform the cloning reactions into Max Efficiency® Stabl2™ competent *E. coli*, following the manufacturer's instructions.
9. Pre-warm the LB plates containing 100 μg/mL of ampicillin (or the required antibiotic).
10. Plate 100 μL of the transformed *E. coli* suspension on LB plates and incubate overnight at 30 °C (between 16 and 24 h).

11. Pick up 5–15 colonies with sterile toothpicks or 20 μL pipette tips.
12. Inoculate each colony into separate 15 mL sterile conical tubes or round-bottom culture tubes containing 5 mL of LB broth plus 100 μg/mL of ampicillin (or the required antibiotic).
13. Incubate overnight at 30 °C, 225 rpm. Loosen the tube caps to allow air into the tubes during incubation.
14. After incubation, purify the plasmids using the plasmid purification kit of your choice, following the manufacturer's instructions.
15. Purified plasmids can be screened by fragment size through DNA gel electrophoresis. The purified plasmids can be run against the original vector plasmid as a control. Plasmids can also be screened by restriction digestion.
16. Plasmids showing the expected size must be confirmed by sequencing analysis to ensure that no unexpected mutations have occurred and to confirm that the insert is in the correct orientation.
17. Once the sequence has been confirmed, the full-length cDNA can be used for virus rescue (*see* **Note 2**).

3.2 Cell Culture Growth and Maintenance

If cells have been kept frozen in liquid nitrogen, follow your source's instructions for thawing. For general growth and care, the following steps are recommended.

1. After extracting a vial of cells from the liquid nitrogen, allow them to thaw.
2. Dispense the contents of the vial into a T25 flask containing pre-warmed DMEM supplemented with 10% FBS and 1× pen icillin/streptomycin solution (complete media).
3. Place the cells into a cell culture incubator set at 37 °C under a 5% CO_2 atmosphere for about 24–48 h.
4. When the cells have reached confluence, remove and discard the media.
5. Wash cells twice with 1 mL of pre-warmed, sterile 1× PBS.
6. Add 0.25% trypsin-EDTA solution, enough to cover the bottom of the T25 flask (between 0.5 and 1 mL).
7. Let incubate for 3–5 min at 37 °C (in the cell culture incubator) until the cells detach. Tap the sides of the flask to help with the detachment.
8. Add an equal volume of complete media to quench the trypsin and wash the bottom of the flask by pipetting.
9. Pipette up and down a few times to resuspend the cells and transfer into a 15 mL conical tube.
10. Centrifuge at 450 × *g* for 5 min.

11. Discard supernatant; add 5 mL of pre-warmed media and pipette up and down until cells have been resuspended. Adjust the volume to 13 mL with complete media.
12. Transfer the cell resuspension to a T75 flask and rock to ensure even distribution of cells.
13. Place flask into the cell culture incubator under the same conditions as above.
14. Pass cells every 3 days.

3.3 Rescue of Lentogenic Newcastle Disease Viruses (Fig. 4)

1. Trypsinize, wash, and count Hep-2 cells manually with hemocytometer or with the automated cell counter.
2. Plate Hept-2 cells at 1×10^6 cells per well in 6-well plates with DMEM supplemented with 5% FBS without antibiotics (2 mL final volume per well). Keep cells overnight (about 16 h) at 37 °C under a 5% CO_2 atmosphere, or until cells have reached 80% and 95% confluency.
3. Wash the cells once with 1 mL of 1× PBS and discard the PBS.
4. Wash once more with 1 mL of Opti-MEM and discard the media.
5. Infect the cells with MVA/T7 at a multiplicity of infection (MOI) of 3 in up to 500 μL of Opti-MEM. Apply carefully in a dropwise fashion so as not to disrupt the cell monolayer. Rock the plates gently to evenly distribute the inoculum.
6. Incubate for 1 h at 37 °C under a 5% CO_2 atmosphere, rocking the plates every 15 min.
7. While the infection is ongoing, prepare the DNA complexes and Lipofectamine mixtures.
   (a) *DNA complexes*: Mix each cDNA with helper plasmids (NP, P, and L) at a ratio of 1:0.5:0.25:0.1 [full-length NDV cDNA (1 μg): NP (0.5 μg): P (0.25 μg):L (0.1 μg)] in 1.5 mL microcentrifuge tubes. After all four plasmid DNAs have been mixed together, bring volume up to 250 μL with Opti-MEM.
   (b) *Lipofectamine*: Gently mix Lipofectamine® 2000 (Invitrogen) by inverting the tube. Mix 4 μL with up to 250 μL of Opti-MEM, mix gently by inverting the tube, and incubate at room temperature for 5 min.
   (c) *DNA-Lipofectamine mixtures*: Add 250 μL of Lipofectamine mixture to each DNA complex, mix gently, and incubate at room temp for 20 min.
8. After cell incubation, discard inoculum, wash the cells once with 1 mL of 1× PBS, and discard.
9. Wash once more with 1 mL of Opti-MEM and discard.

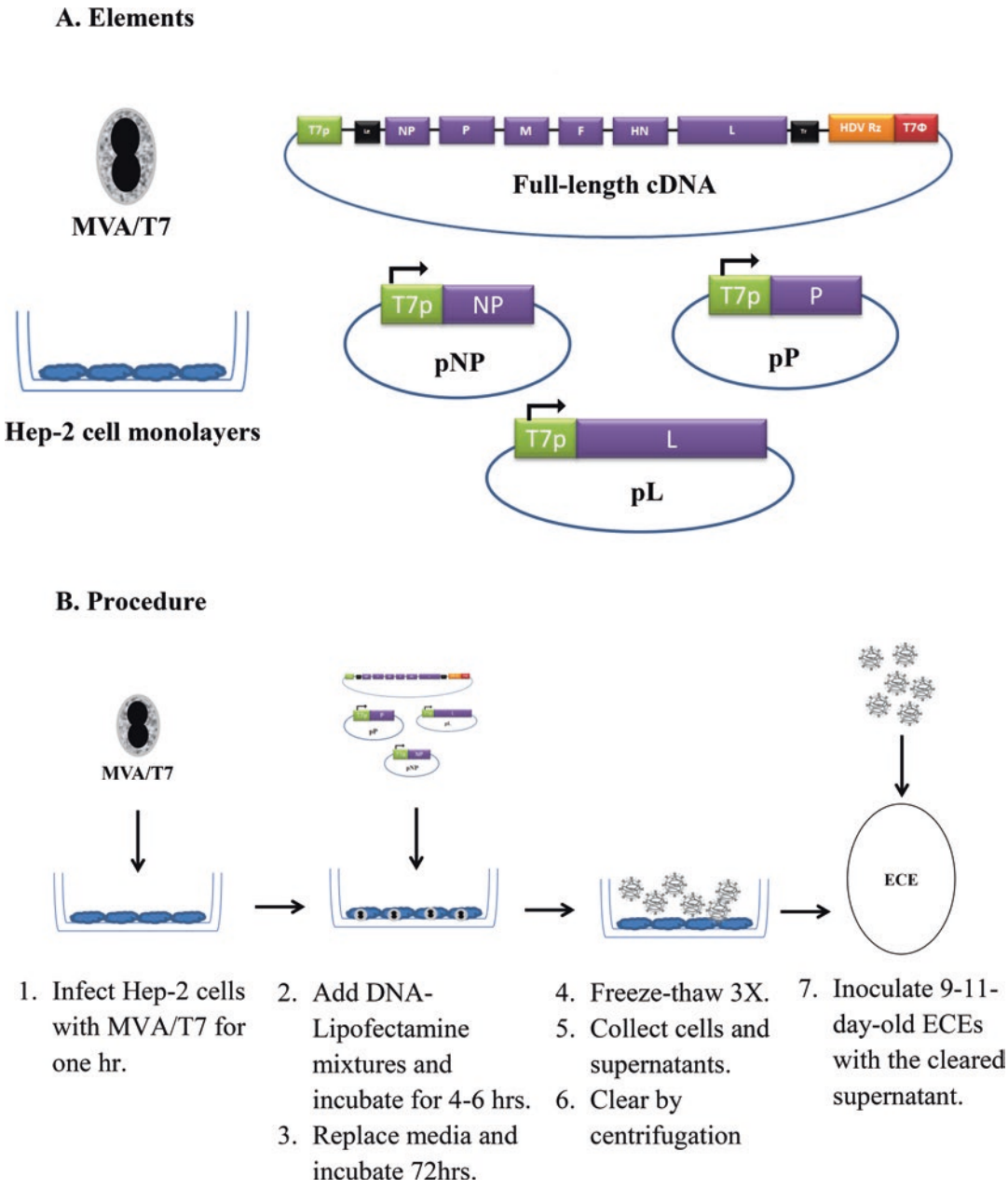

Fig. 4 Schematic of the virus rescue procedure. (**a**) Elements, (**b**) procedure

10. Add 1 mL of fresh Opti-MEM to each well and the DNA-Lipofectamine mixtures in a dropwise fashion to the corresponding wells.
11. Gently rock the plates to evenly distribute the mixtures.
12. Incubate during 4–6 h at 37 °C under a 5% of CO_2 atmosphere.

13. Following cell incubation replace the old media with fresh DMEM supplemented with 1× penicillin/streptomycin solution and porcine pancreatic trypsin (1 μg/mL). *Do not add FBS to the media*.
14. Incubate for 72 h at 37 °C under a 5% CO_2 atmosphere, checking cells daily for cytopathogenic effects.
15. After 72 h, harvest the cells and perform three rapid freeze-and-thaw cycles. This can be achieved using an ultra-freezer and the cell culture incubator.
16. Clear the cell culture supernatants by centrifugation at 1200 × *g* for 10 min at 4 °C.
17. Transfer the cleared supernatants into 1.5 mL microcentrifuge tubes and set on ice for ECE inoculation.

3.4 Rescue of Mesogenic and Velogenic Newcastle Disease Viruses

1. Follow **steps 1–12** from Subheading 3.3.
2. Once the 4–6-h incubation period is done (**step 13** from Subheading 3.3), replace the old media with fresh DMEM supplemented with 2.5% FBS and 1× penicillin/streptomycin solution.
3. Follow **steps 14–17** from Subheading 3.3.

3.5 Propagation of Rescued Viruses in Embryonated Chicken Eggs (ECEs)

1. Candle the ECEs to ensure viability and mark with a pencil the limit between the air chamber and the allantoic cavity.
2. Disinfect the top of the egg shell with ethanol/iodine solution and allow to air-dry.
3. Using the egg puncher or the needle, punch a hole on the egg shell, above the pencil mark.
4. Inoculate three SPF ECEs (9–11 days old) with 300 μL of cleared supernatant. A set of three eggs is required for each supernatant sample.
5. Seal the hole on the egg shell with glue.
6. Place the eggs into an incubator and let incubate for up to 7 days at 37 °C, candling daily for mortality.
7. Chill the eggs overnight at 4 °C after death or when 7 days have passed (whichever occurs first).
8. Collect as much allantoic fluid as possible from each egg into separate 15 mL conical tubes, using the 5 mL syringes and 16G × 1.5″ needles.
9. Perform hemagglutination (HA) test on each allantoic fluid sample. If samples test positive for the HA test, a second passage in eggs can be done to amplify the virus, following the same procedure as before. If the samples do not test positive, subsequent passages in eggs (up to four) are required before considering the virus rescue attempt as unsuccessful.

10. Rescued virus present in the allantoic fluid should be dispensed into 0.5 or 1 mL aliquots, either into cryovials or O-ring screw-cap tubes, and stored at −80 °C.
11. Sequencing analysis is required to confirm the identity of every rescued virus.

4 Notes

1. *Safety considerations*
   (a) According to the Code of the Federal Regulations, Title 9, Chapter I, Sub-chapter E, Part 121.3, nucleic acids that can produce infectious forms of any select agent are subjected to the regulations for select agents. Therefore, these experiments should be conducted in a BSL-3 facility at all times, until rescued viruses are deselected.
   (b) Wear personal protective equipment: lab coat or disposable gowns, safety glasses, and gloves.
   (c) Hep-2 cells contain human papillomavirus; therefore, they should be grown and maintained under BSL-2 conditions (refer to the ATCC website for details).
   (d) Conduct all cell culture work and virus rescue procedures in a biosafety cabinet in order to maintain sterility conditions and reduce pathogen exposure.
2. *Considerations related to genome size*
   (a) Always sequence after each step involving RT-PCR, PCR amplification, or any genetic manipulation of the genome. It is important to confirm that there are no unexpected mutations or deletions, that the intentional mutations were introduced, and that the insert or gene replacements are in the correct location and orientation.
   (b) *The rule of six*: Newcastle disease virus follows the so-called rule of six, which refers to the fact that the NDV genome's length is always a multiple of six. This has to do with the encapsidation process, where the ribonucleoprotein complex molecules bind to six nucleotides at a time. If, for any reason, the length of the genome is not a multiple of six, there is no proper encapsidation and therefore the ability to rescue viable viruses is hampered [42]. This is an important consideration that has to be taken into account during the design and development of the full-length cDNA plasmids.
   (c) *Size of the insert*: It has been reported that insertion of nucleic acids that increase the size of the NDV genome may attenuate the virus, probably by decreasing its replication ability [21, 43]. In addition, due to its non-segmented

genome, the virus has a limited tolerance for carrying multiple or long (>3 kb) transgenes [21]. The largest single gene that has been inserted into the NDV genome is the spike S gene from severe acute respiratory syndrome (SARS), which is 3768 bp [20, 21]. In another attempt to overcome the limitations for insert size, a segmented NDV genome, carrying the spike S gene from SARS and the GFP gene, was developed, showing that the segmented genome facilitated the ability of NDV to carry and express multiple transgenes at a time [21].

(d) *Plaque purification of parental viruses*: A significant precaution to take during functional studies is to plaque purify viruses that will be used to create full-length cDNA plasmids. As all RNA viruses mutate easily, sequencing and cDNA amplification may yield products that represent an average quasispecies rather than a functional virus. This may explain why sometimes scientists cannot fully rescue the phenotype of a wild-type virus by reverse genetics. Our experience demonstrates that utilizing plaque-purified viruses as starting material, in general leads to recombinant viruses with phenotypes that are indistinguishable from wild-type viruses. Plaque purification also helps to eliminate any possibility of mixed virus population with varying genotypes or virulence.

3. *Considerations for vector/insert amplification by PCR*

(a) The use of pfuULTRA™ II Fusion HS DNA polymerase (Stratagene) to amplify the vector/insert before cloning to generate the full-length cDNA plasmid, is recommended since other PCR kits may not produce a linearized vector/insert of enough quality for the In-Fusion cloning technique.

(b) When amplifying/linearizing the vector before cloning of the full-length cDNA plasmid, one may need to prepare multiple PCR reactions to obtain enough DNA for the cloning step. After the DNA gel purification step, multiple vector PCR products can be concentrated into a single tube using a PCR purification kit or through ethanol precipitation.

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Chapter 11

Reverse Genetics Systems for Filoviruses

Thomas Hoenen and Heinz Feldmann

Abstract

Filoviruses are among the most pathogenic viruses known to man. Reverse genetics systems, in particular full-length clone systems, allow the generation of recombinant filoviruses, which can be used to study virus biology, but also for applied uses such as screening for countermeasures. Here we describe the generation of recombinant filoviruses from cDNA.

Key words Ebola virus, Marburg virus, Filoviruses, Reverse genetics, Full-length clone system, Life-cycle modeling system, Infectious clone, Minigenome, trVLP system

1 Introduction

Filoviruses encompass ebolaviruses and marburgviruses, and cause severe hemorrhagic fevers in humans and nonhuman primates [1]. They are among the most pathogenic viruses known to man, with case fatality rates of up to 90%, and are classified as biosafety level (BSL) 4 agents, which restricts research on infectious filoviruses to a small number of maximum containment laboratories worldwide. While filoviruses were discovered almost 50 years ago in 1967 in Marburg, Germany, they are maybe best known from a massive outbreak of Ebola virus hemorrhagic fever (EHF) in 2014/2015 in Western Africa [2], which involved almost 28,000 cases, and caused more than 11,000 deaths (as of September 2015, when the outbreak was still ongoing). Significant progress has been made over the last years in the development of countermeasures, and clinical trials were ongoing in 2015 testing both vaccines and antivirals [3–5]. However, despite this progress there is still an urgent need for a better understanding of filovirus molecular biology, ecology, and pathogenesis, and also for the further development of countermeasures.

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From a molecular biology point of view, filoviruses are non-segmented negative-sense RNA viruses, whose genome encodes seven structural proteins: (1) the nucleoprotein NP, which encapsidates the genome [6]; (2) the polymerase cofactor VP35 [6], which also acts as interferon (IFN) antagonist [7]; (3) the matrix protein VP40, responsible for morphogenesis and budding of progeny virus particles [8], and in case of marburgviruses also an IFN antagonist [9]; (4) GP, the sole surface glycoprotein, which facilitates entry and fusion of virus particles with target cells [10]; (5) VP30, which for ebolaviruses acts as transcriptional activator [6], and is involved in mRNA editing of the ebolavirus glycoprotein mRNA [11]; (6) VP24, a protein involved in the formation of functional nucleocapsids [12, 13], which in case of ebolaviruses also serves as an IFN antagonist [14]; and (7) the viral polymerase L [6]. Structurally, virus particles have a characteristic threadlike appearance, giving rise to the name filoviruses (Latin *filum* = thread). The center of virus particles contains a helical nucleocapsid consisting of the viral RNA genome, NP, VP35, VP30, and L (together known as ribonucleoprotein complex or RNP proteins), as well as VP24 [15, 16]. Surrounding the nucleocapsid is the matrix space containing VP40, which links nucleocapsids with GP, which is embedded in the host cell-derived virus membrane.

Reverse genetics is the generation of viral genomes or genome analogues from cDNA [17]. For filoviruses, reverse genetics systems can be broadly divided into full-length clone systems, which allow the generation of recombinant viruses, and life-cycle modeling systems, which model aspects of the virus life cycle and can be safely used outside of a maximum containment laboratory. In this chapter we focus on the use of full-length clone systems for ebolaviruses (although similar protocols should also be applicable for marburgviruses); for a review of life-cycle modeling systems and detailed instruction for their use the interested reader is referred to [18, 19].

Full-length clone plasmids contain a cDNA copy of a complete filovirus genome, generally in antigenomic or cRNA orientation, and allow expression of this virus cRNA in mammalian cells (Fig. 1). In most cases, this is accomplished with the help of a coexpressed T7 RNA polymerase, although other strategies, such as RNA polymerase II-driven expression of cRNA, are feasible. Coexpressed filovirus RNP proteins are able to recognize the cRNA as an authentic viral template for genome replication, and replicate it into vRNA genome, which in turn can be transcribed into mRNAs, initiating the virus life cycle. Since full-length plasmids can be genetically manipulated with relative ease, this allows for the rescue of recombinant filoviruses, with numerous possible applications.

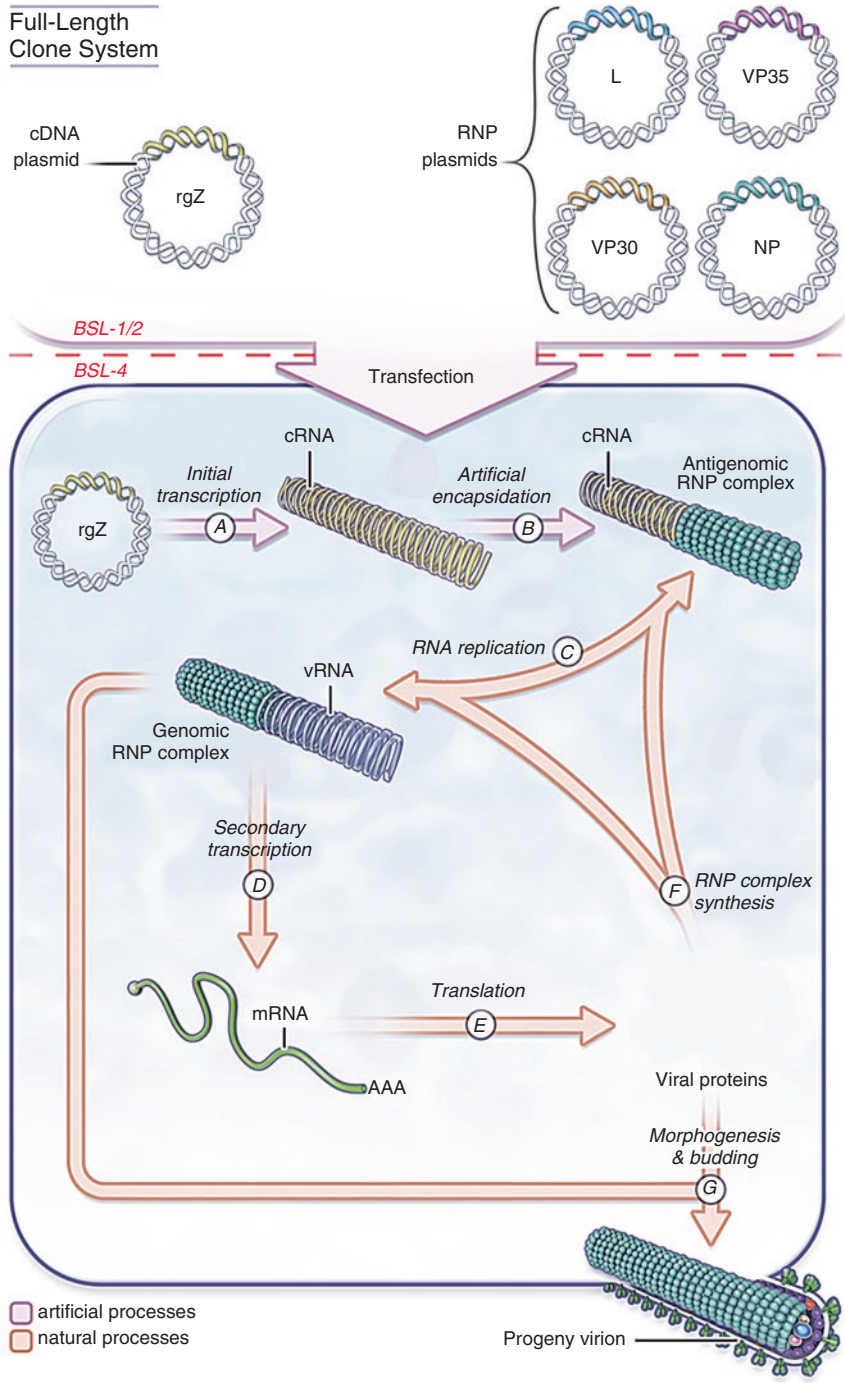

Fig. 1 Schematic of the rescue procedure. Cells are transfected with a full-length plasmid (rgZ) and the RNP plasmids encoding L, VP35, VP30, and NP. Initial transcription (A) either by cellular PolI or PolII or alternatively by T7 RNA polymerase provided from a plasmid (not shown) results in a "naked" full-length genome (cRNA), which is subsequently encapsidated (B) and can then serve as a template for genome replication (C) and transcription (D). This results in translation of viral proteins (E), which further encapsidate newly replicated genomes (F) and lead to the generation of recombinant virus particles (G). Reprinted from [17] with permission from Elsevier

Obviously, these recombinant viruses can be used to study virus molecular biology, and indeed they have been used, for example, to better understand GP-production and processing [20, 21], the role of VP40 and budding mechanisms employed by filoviruses [22, 23], the function of VP30 [24, 25], and details of genome replication, transcription, and translation [13, 26, 27]. Also, studying these viruses can allow us to better understand pathogenesis, for example the contributions of individual genes or gene functions to pathogenicity [28, 29]. Finally, reporter-expressing viruses can be generated (e.g., viruses expressing GFP or luciferases), which can be used as tools for antiviral screening [30, 31]. It is important to point out that while the generation of recombinant viruses and their subsequent characterization of course have to be done in a maximum containment laboratory under BSL4 conditions, the full-length clone plasmids themselves can be safely manipulated in a regular laboratory, although one should consider to keep work with those plasmids physically separated from other plasmids (i.e., RNP protein expression plasmids) in order to eliminate any risk of contaminations and accidental rescue of infectious viruses. In this context it should also be noted that the name "infectious clone" sometimes used for full-length clones is somewhat misleading, since for negative-sense RNA viruses cDNA plasmids are in fact not infectious at all, which is an important reason why they can be safely handled outside of a maximum containment laboratory.

2 Materials

2.1 Rescue and Passaging of Viruses

1. Cells for initial rescue and passaging, e.g., Vero, 293, or Huh7 cells (*see* **Note 1**).
2. Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) or 5% (v/v) fetal bovine serum (FBS, heat-inactivated for 30 min at 56 °C) and 1% l-glutamine (Q, 2 mM) and 1% penicillin/streptomycin (PS, 100 U/mL).
3. 6-well plates and T150 tissue culture flasks.
4. Transit-LT1 transfection reagent.
5. Cell scrapers, 50 mL Falcon tubes, and 1.5 mL cryovials.

2.2 Sequence Confirmation of Rescued Viruses

1. QIAAmp viral RNA mini kit.
2. 96–100% Ethanol.
3. Superscript III with 5× FS buffer and 0.1 M DTT, RNase-free water, RNaseOUT, dNTPs (10 mM each).
4. IProof DNA polymerase.

5. PCR cooler and PCR cycler.
6. PCR purification kit.
7. Primers (*see* **Note 2**): #3185 cggacacacaaaaagaaagaag; #3186 caaatacttgactgcgccac; #3187 gagtgcggacagtttccttc; #3188 gtactcccgtgtgctgtgg; #3189 ggccaagcatggagagtatg; #3190 gttctgtgagggcctgggac; #3191 gggtggacaacagaagaacag; #3192 cctgttttcgttccttgactac; #3193 catggcaatcctgcaacatc; #3194 cagtagccaatgaagccaatg; #3195 cgaagccaaacccgaagac; #3196 caagctcggggaatgtcac; #3197 ctcctcaatgtgccctaattc; #3198 gatgaatgctgatgacacactg; #3199 ggacactccatcgaatccac; #3200 catggtgaggtctcctggag; #3201 gaccggtaagaaggtgacttc; #3202 gttgccccacaatatccttctag; #3203 gcgtaatcttcatctctcttag; #3204 gggtcctccgttgcattgac; #3205 gaaggtgtcgttgcatttctg; #3206 gtcgtggcagagggagtg; #3207 cttgacatctctgaggcaac; #3208 ccatcctgtccaccaattgtc; #3209 cgaaccacatgattggaccaag; #3210 ctcgataattctctctggatgatg; #3211 atatgagagaggacgcccac; #3212 cacacggtaactggagagc; #3213 cagttttgaagctgcactatg; #3214 cttatcagacctccgcattaatc; #3215 gaggtgtttggtattggctattg; #3216 atgcaggggcaaagtcattag; #3217 ggtggaaggtttattgggctg; #3218 cagtgaggatttatctgtggttaaac; #3219 gttatcttgacatctctgctttc; #3220 gagagcatcttgcattgtgtac; #3221 caaggcactgtcaggcaatg; #3222 gggtgtgatttacagctaaatgc; #3223 cctcgaaccattgtgcttgg; #3224 gatattgtggtagtagatactcgag; #3225 cctcacaaatttagtactaaacgtg; #3226 gtctgcgtcagtctctaag; #3227 ctggacaagtatttcatgtgctc; #3228 gcgacccagggacatttaatc; #3229 ctccgaatgattgagatggatg; #3230 ccgatagtccagcttattcg; #3231 caaccaggtgggaaaccattc; #3232 cagccgtttaccttggaaaaatg; #3233 ccgagaaaacgaattgatttatg; #3234 tgtcgtgaggatgtacatgatc; #3235 ggtcaaaacccaacacctgtg; #3236 ccgacttgaaactctctatttc; #3237 gagatccgtcattgataccacag; #3238 agttaaatgacttagccagtatgg; #3239 atgccacaccaaaaccatctc; #3240 cagggagagaggctaaatatag; #3241 cctgatacttgcaaaggttgg; #3242 tggacacacaaaaaagaagaaatag.

3 Methods

3.1 Initial Rescue of Recombinant Viruses

1. Seed p0 cells (*see* **Note 1**) in 2 mL $DMEM_{10\%FCS/PS/Q}$ in 6-well plates for ~50% confluence on the next day. Incubate cells at 37 °C and 5% CO_2 in a humidified incubator.
2. After 24 h, combine helper plasmids (125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 1000 ng pCAGGS-L or pCAGGS-eGFP, 250 ng pCAGGS-T7) in a tube, add 100 μL OptiMem per well, vortex, and spin down. It is advisable to transfect several wells in parallel since rescue efficacy is usually below 100%, and to use one well as negative control by replacing pCAGGS-L with an equal amount of pCAGGS-eGFP.

3. Transfer cells, diluted helper plasmids, and full-length clone plasmid into a BSL4 laboratory.
4. Add 250 ng full-length plasmid to diluted helper plasmids.
5. Briefly vortex vial with Transit-LT1. Add 5.5 µL (*see* **Note 3**) Transit LT1 per well to the mix, vortex, and spin down. Incubate for 15 min at room temperature to allow transfection complexes to form.
6. After 15 min, add 100 µL of transfection complexes in a dropwise fashion to the cells, trying to cover the whole surface area of the well.
7. Rock plates back and forth and from side to side. Do not swirl plates, to avoid transfection complexes being pushed to the edge of the well. Return cells to the incubator.
8. At 24 h post-transfection, replace the medium with 4 mL $DMEM_{5\%FCS/PS/Q}$ (*see* **Notes 4** and **5**). Return cells to the incubator.

3.2 Passage of Recombinant Viruses

1. Seven days post-transfection, seed p1 cells (*see* **Note 6**) in 4 mL $DMEM_{10\%FCS/PS/Q}$ in 6-well plates for ~90% confluence on the next day. Incubate cells at 37 °C and 5% CO_2 in a humidified incubator.
2. On the next day (i.e., 1 week after transfection), take p1 cells into the BSL4 laboratory. Add 1 mL of supernatant from p0 cells to p1 cells (*see* **Note 7**).
3. From now on, check for cytopathic effect (CPE) in p1 cells daily (*see* **Note 8**). If reporter-expressing viruses (e.g., viruses expressing GFP or luciferase) are being rescued, reporter activity can be used as an alternative readout.
4. Once clear CPE is visible, passage 1 mL of supernatant onto a T150 flask with 90% confluent cells (p2 cells) in 60 mL $DMEM_{10\%FCS/PS/Q}$ (*see* **Note 7**). Check for CPE in p2 cells daily.

3.3 Harvest of Recombinant Viruses

1. Once p2 cells show clearly discernibly CPE, harvest virus. To do so, scrape cells into the medium using a cell scraper, transfer 30 mL cell suspension each into two 50 mL Falcon tubes, and spin down for 10 min at 1000 × *g* at 4 °C.
2. Pour clarified supernatant into Falcon tubes, each containing 3 mL FBS (heat-inactivated for 30 min at 56 °C), mix by inversion, and aliquot. Store aliquots in liquid nitrogen.

3.4 Sequence Confirmation of Rescued Virus

1. To ensure a correct sequence of the rescued virus (*see* **Note 9**), inactivate 140 µL virus stock by adding it to 560 µL AVL buffer (from the QIAamp viral RNA mini kit). Vortex and incubate for 10 min.
2. Remove inactivated virus from the biosafety cabinet, and transfer it into a clean tube containing 560 µL 96–100% ethanol.

Mix by vortexing, and then remove the sample from the BSL4 laboratory following appropriate protocols.

3. Extract viral RNA using the QIAamp viral RNA mini kit, following the manufacturer's instructions.
4. In a PCR tube, combine 1 μL RNA, 1 μL RT primer #3185 (2 μM), 1 μL dNTPs, and 9 μL RNase-free water (*see* **Note 10**). Incubate for 5 min at 65 °C, and then place immediately on ice for at least 1 min.
5. Meanwhile, combine 4 μL 5× FS buffer, 1 μL 0.1 M DTT, 1 μL RNaseOUT, and 1 μL SuperScript III; vortex briefly; and place on ice for at least 1 min.
6. Add 7 μL of this mix to the RNA/primer/dNTP mix (*see* **Note 9**), incubate for 50 min at 50 °C, then heat-inactivate for 5 min at 85 °C, and cool down to 4 °C.
7. Add 1 μL RNase H (*see* **Note 10**), incubate for 20 min at 37 °C, and then store cDNA at 4 °C.
8. Label 24 PCR tubes (e.g., three 8-tube strips) and place in prechilled PCR cooler.
9. Prepare primers (Table 1, 5 μM each) in a 96-well format (*see* **Note 11**).
10. Program PCR cycler (Table 2) and preheat to 98 °C.
11. Prepare PCR-mastermix by combining 773.75 μL water, 250 μL 5× GC buffer, 25 μL dNTPs (10 mM each), 37.5 μL DMSO, 12.5 μL DMSO, and 6.25 μL IProof polymerase (all from the IProof kit, Biorad); vortex; and place on ice (*see* **Note 12**).
12. Add 1105 μL PCR-mastermix to 20 μL cDNA, vortex, and pipet 45 μL into each PCR tube.
13. Add 5 μL primers using a multichannel pipette, mix (*see* **Note 10**), and cycle reactions in PCR cycler.
14. After the run, PCR-purify the products using a commercial PCR purification kit following the manufacturer's instructions, e.g., the Machery-Nagel PCR cleanup kit (*see* **Notes 13** and **14**).
15. Sanger-sequence the purified PCR products using the primers listed in Table 1.

4 Notes

1. Various cell lines and cell line mixtures have been used for the rescue of recombinant filoviruses, e.g., 293/Vero mix, Vero cells, or Huh-7 cells. These cell lines vary in their susceptibility for filoviruses and their transfectability, both of which impact rescue efficiency. For example, 293 and 293T cells are highly transfectable, but only poorly susceptible to filovirus infection; however, this can be overcome by expressing the virus adhesion

Table 1
Primer combinations for PCRs and subsequent sequencing

Forward PCR primer	Reverse PCR primer	Product size (kB)	Primers for sequencing
#3185	#3186	~0.7	#3185, #3186
#3187	#3192	~2.1	#3187, #3188, #3189, #3190, #3191, #3192
#3193	#3198	~2.1	#3193, #3194, #3195, #3196, #3197, #3198
#3199	#3204	~2.1	#3199, #3200, #3201, #3202, #3203, #3204
#3205	#3210	~2.1	#3205, #3206, #3207, #3208, #3209, #3210
#3211	#3216	~2.1	#3211, #3212, #3213, #3214, #3215, #3216
#3217	#3222	~2.1	#3217, #3218, #3219, #3220, #3221, #3222
#3223	#3228	~2.1	#3223, #3224, #3225, #3226, #3227, #3228
#3229	#3234	~2.1	#3229, #3230, #3231, #3232, #3233, #3234
#3235	#3240	~2.1	#3235, #3236, #3237, #3238, #3239, #3240
#3241	#3242	~0.7	#3241, #3242
#3185	#3190	~2.1	#3185, #3186, #3187, #3188, #3189, #3190
#3191	#3196	~2.1	#3191, #3192, #3193, #3194, #3195, #3196
#3197	#3202	~2.1	#3197, #3198, #3199, #3200, #3201, #3202
#3203	#3208	~2.1	#3203, #3204, #3205, #3206, #3207, #3208
#3209	#3214	~2.1	#3209, #3210, #3211, #3212, #3213, #3214
#3215	#3220	~2.1	#3215, #3216, #3217, #3218, #3219, #3220
#3221	#3226	~2.1	#3221, #3222, #3223, #3224, #3225, #3226
#3227	#3232	~2.1	#3227, #3228, #3229, #3230, #3231, #3232
#3233	#3238	~2.1	#3233, #3234, #3235, #3236, #3237, #3238
#3239	#3242	~1.4	#3239, #3240, #3241, #3242
#3197	#3198	~0.7	#3197, #3198
#3209	#3212	~1.4	#3209, #3210, #3211, #3212
#3213	#3216	~1.4	#3213, #3214, #3215, #3216

factor Tim-1 in these cells [13, 32]. In contrast, Vero cells are highly susceptible to filovirus infection, but somewhat harder to transfect. Further, recent work has shown that the choice of cell line seems to influence the sequence fidelity with which recombinant viruses can be rescued, with rescue in Vero cells resulting in certain mutations (particularly A insertions in poly-A stretches) in the genome with higher frequency than when using other cells [33]. These properties should be taken into

Table 2
PCR conditions

Step	Duration	Temperature	Remarks
1	30 s	98 °C	
2	15 s	98 °C	
3	30 s	59–54.5 °C	Reduce temperature by 0.5 °C per cycle
4	60 s	72 °C	
5			Return to step 2 for a total of 10 cycles
6	15 s	98 °C	
7	30 s	54 °C	
8	60 s	72 °C	
9			Return to step 6 for a total of 30 cycles
10	3 min	72 °C	
11	Hold	4 °C	

consideration when choosing a cell line for rescue; the authors would recommend Huh-7 cells for initial rescue, and either Huh-7 or Vero cells for passaging.

2. All primer sequences provided here are for the species *Zaire ebolavirus* [34].
3. The amount of Transit-LT1 might need to be adjusted to obtain optimal transfection efficacy. As a starting point for most cell types, a ratio of about 3 μL Transit-LT1 per 1 μg DNA should be used; for example, for Vero cells this ratio should be increased to about 6:1.
4. At this point, the supernatant should be considered to contain infectious ebolaviruses, and standard practices should be used to avoid cross-contaminating wells (i.e., changing of pipettes between different wells).
5. It is paramount at this point to completely remove all supernatant prior to adding fresh medium, in order to remove traces of the cDNA plasmid which could cause problems in downstream procedures. This can be achieved by first removing the supernatant with a serological pipette, and then removing any remaining liquid with a P1000 pipette.
6. Again the choice of cells can be varied. Most commonly used at this point are Vero cells; however, continuous passaging in these cells can lead to nucleotide insertions in regions encoding poly-A stretches, for example at the GP gene-editing site [33, 35].
7. An alternative approach is to freeze 1 mL of p0 supernatant at −80 °C at the same time the p0 supernatant is passaged to p1

cells in a 6-well format. Once p1 cells show clear CPE, the corresponding p0 supernatant can be used to inoculate the T150 for stock growth. This reduces the passage number for the virus stock by one, which reduces the chance of appearance of unwanted mutations, while still allowing for easy screening of a larger number of wells for successful virus rescue.

8. The onset of CPE is dependent on the properties of the rescued virus, as well as the rescue efficacy. As a rule of thumb, clear CPE (compared to the -L control) should start to be visible about a week after passaging.
9. Given the tendency of rescued ebolaviruses to show unwanted mutations, it is imperative that they are completely sequenced prior to use.
10. To ensure proper mixing, tubes should be flicked repeatedly and then briefly spun down.
11. If sequencing is done more often, primers can be frozen in a 96-well format using 8-tube strips. In this case, make sure that strips are spun down thoroughly before opening, and label both strip caps and tubes, to avoid cross-contamination. Strips from some manufacturers are difficult to open, resulting in an increased risk of spilling the contents, and can be incubated empty at 99 °C in a PCR cycler for 5 min before they are first used to alleviate this issue.
12. Amounts are for 24 + 1 reactions, i.e., sufficient to sequence one virus completely. If more than one virus is supposed to be sequenced, the amount can be scaled up; however, since the IProof polymerase has a 3′–5′ exonuclease activity and does not have to be heat-activated, it becomes paramount that all reactions are kept on ice or in a PCR cooler.
13. If desired, PCR products can be visualized using standard gel electrophoresis prior to purification. In this case, loading 2 μL of PCR product should result in easily visible bands. Expected product sizes can be found in Table 1.
14. Other PCR purification kits can also be used; however, the Macherey Nagel kit allows to use diluted buffer NTI (1:4 in water) for the initial DNA binding step, which decreases the amount of small, unspecific products (primer-dimers) in the purified DNA.

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Chapter 12

Rapid Reverse Genetics Systems for Rhabdoviruses: From Forward to Reverse and Back Again

Tobias Nolden and Stefan Finke

Abstract

Methods to recover recombinant negative strand RNA viruses (rNSVs) from cloned cDNAs have been significantly improved in more than two decades of NSV reverse genetics. In particular, for non-segmented negative strand RNA viruses (NNSVs) like rhabdoviruses, time-consuming generation of reverse genetics systems by stitching PCR subfragments of genomic rhabdovirus cDNAs using ligase-based conventional cloning approaches limited the number of available recombinant virus cDNA clones. As genetic variability is considered an intrinsic feature of RNA viruses, it is thus reasonable to conclude that reverse genetics approaches to investigate natural virus functions and pathogenesis require improved systems that reflect the complexity of naturally occurring wild-type viruses, and that largely exclude adaption to cell culture conditions.

In order to allow rapid cloning of wild-type NSV genome populations into reverse genetics vector plasmids, we developed a system in which cDNA copies of complete rhabdovirus populations are inserted into a plasmid bank by linear-to-linear homologous RecE/T recombination (LLHR). Limited requirements for sequence information a priori, high cloning efficiencies, and the possibility to directly generate recombinant viruses from individual cDNA clones now offer novel opportunities to combine forward genetic dissection of natural rhabdovirus populations and downstream reverse genetics approaches.

Key words Rhabdovirus, Rabies virus (RABV), Reverse genetics, Recombination, RecE/T recombinase

1 Introduction

The history of negative strand RNA viruses (NSVs) reverse genetics systems is intrinsically tied to the rhabdovirus family, as recombinant Rabies Virus (RABV) and Vesicular Stomatitis (VSV) represent the first examples of NSVs recovered from cloned cDNA [1–5]. Although rather inefficient in the beginning, those systems revolutionized rhabdovirus and other NSV research by allowing for the first time targeted introduction of genetic modifications and subsequent analysis of phenotypic changes (for review *see* [6]). The reverse genetics strategies were highly complementary to conventional virus genetics, which relied on "forward" approaches (Fig. 1) by correlation genetic variations with an observed

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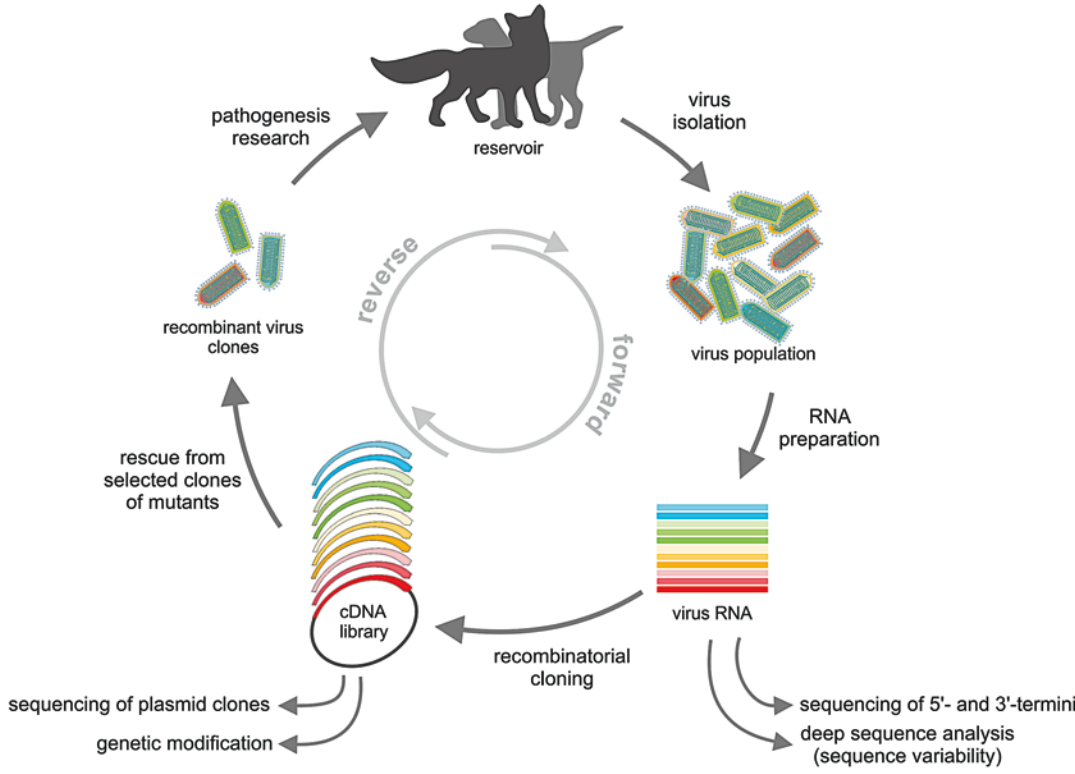

Fig. 1 From forward to reverse to forward. Viruses become visible and are isolated because of visible pathogenicity (phenotypes) in natural reservoir species. Isolation from natural reservoirs without extensive cell culture adaptation is expected to yield a heterogenous virus population. Genomic RNA reflecting the genetic variability in the virus genomes is prepared from the virus population, and cDNA copies generated by complete genome PCRs are inserted into reverse genetics plasmid vectors by recombinatorial cloning to yield a cDNA library that reflects the genetic variability of the progenitor virus population. After sequence analysis on the clonal level selected variants or genetically modified derivatives thereof are directly used for reconstitution of "recombinant field viruses" to allow downstream reverse genetics approaches. Because of the high efficiency of the system, subsequent second or third rounds of recombinatorial cloning and virus reconstitution can be performed

phenotype or mutational analysis of individual genes, frequently outside of the remaining virus context.

Although reverse genetics systems are more or less routinely used, to discover virus gene functions, determinants of virus pathogenesis or to develop novel virus vaccines, limitations exist because of the clonal character of available full-length cDNA plasmids. As sequence variability in natural virus populations is considered an intrinsic feature of RNA viruses, it is reasonable to conclude that an individual cDNA clone and virus generated thereof cannot represent the whole virus population. Moreover, many reverse genetics systems are based on cell culture or animal model adapted laboratory strains, which in case of RABV are dubbed "fixed viruses." Adaptive mutations in such clones, loss of virulence in

relation to the progenitor wild-type virus isolate to an unknown degree, often make it difficult to correlate experimental findings with the situation in the naturally infected host.

We recently addressed the above-described bottleneck by the development of a highly efficient pipeline to clone multiple complete wild-type rhabdovirus genome cDNAs directly into reverse genetics plasmid vectors, with limited requirements for a priori virus sequence information [5]. Because of the systems high efficiency, the approach allowed a true forward to reverse genetics approach including selection of wild-type viruses according to pathogenic properties in carnivores and murine models [7] and deep sequence analyses of progenitor wild-type virus populations. Most importantly, generation of a full-length virus cDNA plasmid library combined with direct use of library plasmids for the generation of recombinant virus clones let to genetically targetable pathogenic, not cell culture adapted "recombinant wild-type virus clones" offering novel opportunities in rabies pathogenesis research.

The here-described protocol for the straightforward generation of reverse genetics systems has been developed for non-cell culture adapted, highly pathogenic wild-type RABV isolated from fox and dog [7] and is considered to be translatable to other NNSVs as long as long-range PCR protocols for complete virus genome cDNA amplification can be established.

With a 12 kb genome encoding the five virus proteins nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, and the large polymerase protein L, RABV is non-segmented and belongs to the lyssavirus genus within the *rhabdoviridae* family [8]. The initial RABV rescue system [1] relied on a bacteriophage T7 RNA polymerase vaccinia helper virus that allowed cytoplasmic T7 polymerase transcription of full-length antigenome RABV vRNAs in cDNA plasmid transfected baby hamster kidney (BHK) cells. After autocatalytic processing of the T7-transcript by a 3′-located hepatitis delta virus ribozyme sequence and co-expression of virus protein N, P, and L from so-called support plasmids, ribonucleoprotein assembly and initial virus replication and transcription events were initiated, leading to further autonomous replication of the recovered virus (reviewed in [6]). Rescue efficiency was highly improved by vaccinia virus free rescue systems, e.g., by the use of genetically modified BHK cell line BSR T7/5 stably expressing T7-polymerase from the genome [3, 9]. Insertion of a 5′-located synthetic hammerhead ribozyme sequence for autocatalytic generation of a correct 5′-end of the transcribed virus RNA also allowed the use of cellular RNA polymerase II promoters for lyssavirus recovery [10, 11]. The here-described reverse genetics vectors include both, a cytomegalovirus immediate early promoter (CMVie) and a T7-promoter, followed by a RABV-specific synthetic hammerhead ribozyme sequence used for RABV minigenome [12] and full virus [4] rescue. As already present in the initial rescue

systems [1], a hepatitis delta virus ribozyme sequence followed by a T7-terminator sequence is located downstream of the full-length virus cDNA sequence. To initiate RABV replication from T7-transcribed vRNA N, P, and L proteins derived from the attenuated vaccine strain SAD B19 [13] were expressed from pCAGGS-based eukaryotic expression plasmids [4] containing a β-chicken actin promoter combined with a CMV enhancer sequence [14]. Importantly, the N, P, and L proteins of the attenuated vaccine strain are able to support rescue of other RABV isolates or strains [5], making it unnecessary to clone autologous support plasmids for each targeted virus isolate.

We here achieve recombinatorial cloning of complete full-length virus cDNA copies into reverse genetics plasmid vectors by RecE/T-mediated linear-to-linear homologous recombination (LLHR). Whereas conventional recombinatorial cloning strategies through lambda phage-derived Redα/Redβ require a replicating circular plasmid backbone (linear-to-circular recombination) and co-integration of a selection marker for DNA insertions [15, 16], linear-to-linear recombinant between two linear DNA fragments with the RAC phage-derived homologous proteins RecE and RecT [17] only requires primer-derived homology sequences (homology arms). Importantly, no additional selection marker must be introduced as only the circularized recombination products are able to replicate in *E. coli*.

The overall procedure of RABV cloning and rescue is a multistep process which can be divided into five parts: (1) isolation of viral genomic RNA from RABV-infected cells by standard procedures, (2) determination of terminal virus sequences by RNA circularization, RT-PCR amplification, and DNA sequencing, (3) long-range RT-PCR amplification of the RABV genome, (4) preparation of the reverse genetics cloning vector and RecE/T-mediated LLHR, and (5) recovery of infectious rRABV clones from recombination products by standard protocols.

2 Material

2.1 Determination of Terminal Virus Sequences

1. 0.2 µl PCR tubes, Biorad.
2. RNA 5′ Pyrophosphohydrolase (RppH), NewEnglandBiolabs.
3. T4 RNA Ligase, NewEnglandBiolabs.
4. Recombinant RNasin® Ribonuclease Inhibitor, Promega.
5. SuperscriptIII Reverse Transcriptase, Invitrogen.
6. GoTaq DNA Polymerase, Promega.
7. QIAquick Gel Extraction Kit, QIAGEN.

Table 1
Oligonucleotides used for RABV end-sequencing, RABV long-range PCR, and amplification of pHaHdmin reverse genetics cloning vector with RABV-specific homology arms

Primer	Sequence	Comment
RABV end-sequencing		
N(93)-rev	ATCTAGCATGCCTGATTATTGACTTTGA	
L(11216)-for	TACTAGCATGCAATCCTTACAATGAGATGA	
RABV long-range PCR		
B19-for	TCGATCCCGGGTC**ACGCTTAACAACCAGATCA**	Leader/ trailer in bold
B19-rev	TAATACACCTGCCCATGCCGACCC**ACGCTTAACAAATAAACAA**	
RABV-for	TCGATCCCGGGTC**ACGCTTAACAACAAAATCA**	
RABV-rev	TAATACACCTGCCCATGCCGACCC**ACGCTTAACAAAAAAACAA**	
Amplification of pHaHdmin with RABV-specific homology arms		
L16 trailer	**TTTGGTTGTTTGATTGTTTTTCTCATTTTTGTTGTTTATTTG TTAAGCGT**GGGTCGGCATGGCATCTCCAC	Homology arms in bold
L16 leader	**GCTTTGCAATTGACAATGTCTGTTTTTTCTTTGATCTGGTT GTTAAGCGT**GACCCGGGACTCCGGGTTTCGTC	
Dog trailer	**TcTGGcTGcTTGATTGTTTTTtcCATcTTTaTTGTTTtTTTGTT AAGCGT**GGGTCGGCATGGCATCTCCAC	
Dog leader	**GCTTTGCAAcTGACgcTGTCTGcTTcTTCTcTGATtTtGTTGTT AAGCGT**GACCCGGGACTCCGGGTTTCGTC	
Fox trailer	**TcTGGcTGTTTGATTGTTTTTtcCATcTTTaTTGTTTtTTTGTT AAGCGT**GGGTCGGCATGGCATCTCCAC	
Fox leader	**GCTTTGtAAcTGACgcTGTCTGcTTcTTCTcTGATtTtGTTGTT AAGCGT**GACCCGGGACTCCGGGTTTCGTC	

Bold: virus isolate specific terminal leader (Le) and trailer (Tr) coding sequences used as "homology arms" for linear to linear recombination. Single nucleotide polymorphisms referring to SAD L16 are indicated by lower case letters

8. BigDye Terminator v3.1 Cycle Sequencing Kit, LifeTechnologies.
9. RABV-specific oligonucleotides (for sequences *see* Table 1).
10. Thermal Cycler C1000, Biorad (or equivalent).
11. ABI genetic analyzer, Applied Biosystems.

2.2 Full-Length Genome RT-PCR

1. 0.2 µl PCR tubes, Biorad.
2. SuperscriptIII Reverse Transcriptase, Invitrogen.
3. Phusion HighFidelity Polymerase, ThermoFisher Scientific.
4. Recombinant RNasin® Ribonuclease Inhibitor, Promega.
5. Nuclease-free water, ThermoFisher Scientific.
6. QIAquick Gel Extraction Kit, QIAGEN.
7. RABV-specific oligonucleotides (for sequences *see* Table 1).
8. Thermal Cycler C1000, Biorad (or equivalent).

2.3 Preparation of the Reverse Genetics Cloning Vector

1. Nuclease-free water, ThermoFisher Scientific.
2. GoTaq DNA Polymerase, Promega.
3. QIAquick Gel Extraction Kit, QIAGEN.
4. DpnI, New England Biolabs.
5. pHaHdmin vector [5], available from authors on request.
6. pHaHdmin-specific oligonucleotides with RABV-specific homology arms (for sequences *see* Table 1).
7. Biorad Thermal Cycler C1000, Biorad (or equivalent).

2.4 Recombinatorial Virus cDNA Cloning by LLHR

1. *E. coli* strain GB05-dir, GeneBridges.
2. L-Arabinose, Sigma Aldrich.
3. Ampicillin sodium salt, Sigma Aldrich.
4. Distilled water cell culture grade, ThermoFisher Scientific.
5. MicroPulser™ Cuvette (1 mm gap), Biorad.
6. LB Broth and LB agar plates, prepared according to standard procedures described elsewhere.
7. Eppendorf® Thermomixer® R, Eppendorf.
8. Refrigerated Bench-top Centrifuge 5427 R, Eppendorf.
9. MicroPulser™ Electroporator, Biorad.

3 Methods

3.1 Isolation of Viral RABV RNA

Virus RNA is isolated from supernatant virions or total RNA from virus-infected cells by standard procedures. In case of field virus isolates with low infectious virus titers in cell culture supernatants, RNA for complete genome RT-PCR amplification is preferably isolated from infected cell cultures as it may lead to more reliable results in downstream RT-PCR amplifications. For genome circularization and terminal virus sequence determination, we recommend RNA isolation from supernatant virions.

3.2 Determination of Terminal Virus Sequences

As 50 nucleotides (nt) homology arms (*see* Fig. 3a green and red arrows) are required for efficient LLHR, the terminal sequences of the isolated virus RNA are determined by genome RNA circularization, RT-PCR amplification of ligated trailer and leader RNA coding sequences and subsequent Sanger sequencing of the resultant PCR product. RNA circulation involves removal of the 5′-phosphates at the viral end by a RNA 5′-Pyrophosphohydrolase (RppH) and ligation by T4 RNA Ligase. Both reactions can be done in two subsequent reactions described elsewhere [5] or as a single tube reaction (*see* Fig. 2b) described here. Both methods can be efficiently used for circularization and subsequent sequencing (Fig. 2).

1. For virus RNA dephosphorylation and circularization set up the following reaction components in a 0.2 µl reaction tube: 400 ng vRNA from supernatant virions, 0.5 µl RNasin (40 U/µl), 2 µl 10× T4 RNA Ligase Puffer (500 mM Tris–HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.5), 1 µl ATP (10 mM), 1 µl T4 RNA-Ligase` (10 U/µl), 1 µl RNA 5′-Pyrophosphhydrolase (5 U/µl), ad 20 µl ddH_2O.
2. Incubate at 37 °C for 1 h in a thermal cycler.
3. Inactivate Ligase reaction by adding EDTA to a final concentration 5 mM and incubate at 65 °C for 15 min.
4. Set up a RT reaction according to the reverse transcriptase manufactures recommendations. Use 5 µl of the RNA ligase reaction. For priming the RT reaction we recommend the oligonucleotide L(11216)-for listed in Table 1 that binds within the L gene and works for most genotype 1 RABV.
5. Amplify DNA with oligonucleotides flanking the ligation site to a PCR product that comprises the trailer and leader RNA coding sequences (*see* Table 1). We recommended to use GoTaq® DNA Polymerase (Promega) but any other Taq polymerase will work accordingly.
6. Purify PCR product after conventional agarose gel electrophoresis using an conventional column-based purification system (QIAquick Gel Extraction Kit) or any other comparable purification Kit.
7. Determine the nucleotide sequence of the PCR product by Sanger sequencing with the same oligonucleotides used for PCR amplification using the BigDye Terminator v3.1 Cycle Sequencing Kit (*see* **Note 1**).
8. Analyze virus end-sequences and use the sequence information to design the homology arms needed the amplification of the RABV reverse genetics vector pHaHdmin for RedE/T recombination. In addition design isolate specific primers for complete genome RT-PCR amplification in case of sequence variations to primers listed in Table 1 are detected.

3.3 Full-Length Genome RT-PCR.

For amplification of complete virus genome cDNAs, the 12 kb RABV RNA is reverse transcribed by a terminal positive sense primer (Table 1) designed according to the individual virus end-sequences (*see* Fig. 2c). Together with a reverse negative sense primer that hybridizes to the opposite genome terminus, a PCR reaction is formed at conditions optimized for long-range PCR.

In case of RABV, a relative high degree of sequence similarity may allow the use of terminal primers for various isolates. Here identical primers were used for two different field virus isolates independently isolated from dog and fox reservoirs. For amplification of a more distant attenuated lab strain (SAD B19), modified

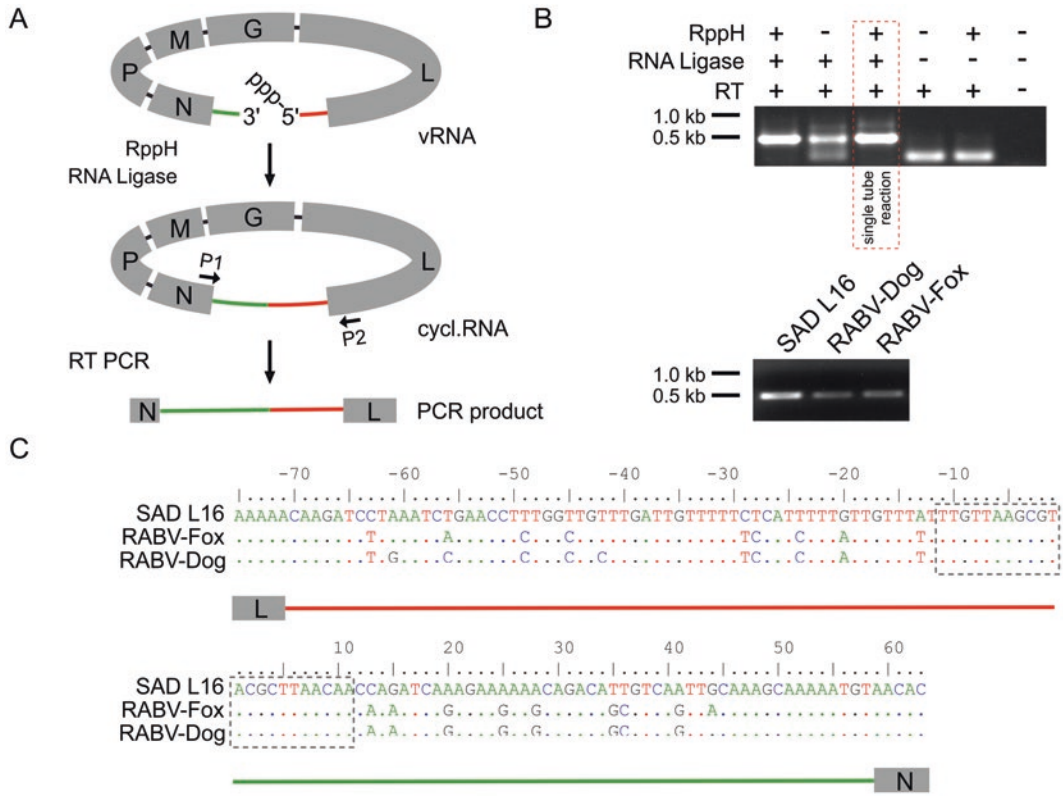

Fig. 2 Determination of virus leader and trailer sequences by RNA T4 ligase-mediated end-joining. (**a**) Schematic presentation of T4-RNA-mediated RNA end-joining for determination of virus end-sequences. The oligonucleotide L16endseq (P2) was used for priming the reverse transcriptase reaction before amplification and sequencing of the PCR product spanning the RNA ligation site. (**b**) RT-PCR products of T4-RNA ligase-mediated end-joining. Positive RT PCR signals were obtained, when RppH digestion was performed as separate reaction or as single tube reaction together with T4-RNA ligase (lane 1 and lane 3, respectively). Without removing 5′ triphoshate, RNA end-joining was less effective (lane 2). No PCR products were obtained when T4 RNA ligase or reverse transcriptase was absent (lane 4, 5 and lane 6, respectively). (**c**) Sequence alignment of SAD L16, RABV-Dog, and RABV-Fox end-sequences. For reference SAD B19 leader and trailer sequences were concatenated. Figure and legend reproduced from Nolden et al., 2016 by Creative Commons Attribution 4.0 International License [5]

primers were used (Table 1). After full-genome cDNA amplification, gel purification of resultant 12 kb products (Fig. 3b) is recommended to avoid recombinatorial insertion of smaller DNA fragments. Such fragments may arise because of internal primer binding or presence of defective interfering RNAs.

1. For long-range RT set up the following reaction components in a 0.2 µl reaction tube on ice: 1 µg total RNA preferentially isolated from RABV-infected cells, 0.5 µl RABV-specific sense primer (10 µM), 0.5 µl RNAsin (40 U/µl), 1 µl dNTPs (10 mM each), and 13 µl nuclease-free H_2O.

2. Mix by flipping the tube and spin down.
3. Incubate in the thermal cycler at 65 °C for 5 min.
4. Place reaction immediately on ice and incubate for at least 1 min.
5. Ad 4 µl 5× RT reaction buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3, 1 µl DTT (0.1 M)) and 2 µl SuperscriptIII reverse transcriptase (200 U/µl). Mix by pipetting up and down a few times.
6. Incubate in the thermal cycler at 55 °C for 60 min.
7. Stop reaction at 70 °C for 15 min.
8. Proceed with PCR (**step 9**) or store cDNA at 4 °C.
9. Set up the following reaction components in a 0.2 µl reaction tube on ice: 0.25 µl cDNA from **step 8**, 10 µl 5× Phusion HF buffer (containing 7.5 mM $MgCl_2$), 1.0 µl dNTPs (10 mM each), 0.5 µl *Phusion*-Polymerase (2 U/µl), 1 µl forward sense primer (10 mM), 1 µl reverse antisense primer (10 mM), and 50 µl nuclease-free H_2O.
10. Mix by flipping the tube and spin down and place tube in the thermal cycler.
11. After 30 s initial denaturation, perform 35 thirty cycles of PCR amplification with the following temperature profile: 10 s denaturation at 98 °C, 15 s annealing at 57–62 °C, 360 s (30 s/kb) elongation at 72 °C. Incubate another 10 min at 72 °C for final extension. The optimal annealing temperature may vary depending on the sequences of used oligonucleotides.
12. Check PCR product by standard agarose gel electrophoresis and purify the full-genome PCR product (in case of RABV 12 kb) from gel.

3.4 Preparation of the Reverse Genetics Cloning Vector

The linear-to-linear recombination requires homologous 50 nt overhangs that allow recombination between the two DNA fragments. As only the vector part contains an antibiotic resistance gene and no marker restriction is used for selection of insert positive plasmids, it is crucial for the method that all circular plasmid copies are removed or destroyed prior to recombination in *E. coli*.

1. Oligonucleotide design:

   Choose 50 nucleotides of the virus 3′ terminus (*see* Fig. 3a, green arrow). Order oligonucleotide with these 50 nucleotides at its 5′-end. The 3′-end of the oligonucleotide contains a sequence that hybridizes to the synthetic hammerhead-ribozyme sequence (Ha) in the reverse genetics vector plasmid pHaHdmin (5′-GACCCGGGACTCCGGGTTTCGTC-3′). Similarly, the 50 terminal nucleotides at the 5′-virus genome end (*see* Fig. 3a red arrow) are present in a second oligonucleotide

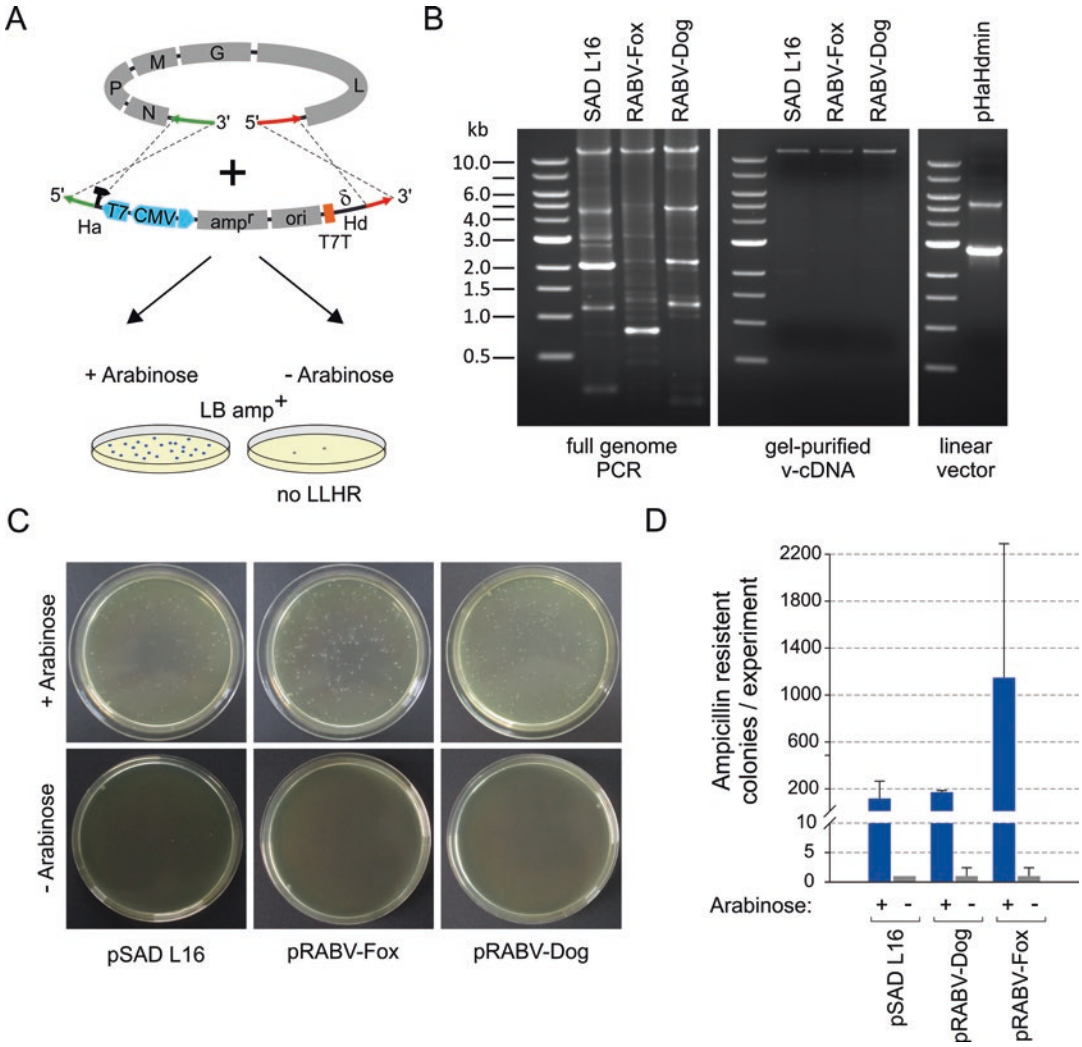

Fig. 3 LLHR of virus genomic cDNA and pHaHdmin linear vector DNA. Schematic presentation of the performed LLHR experiment. Linear full-length genome PCR products from three different rabies viruses (attenuated cell culture adapted SAD L16 and field viruses isolated from dog or fox) and PCR-amplified linear vector DNA were electroporated into arabinose induce or non-induced *E. coli* Gb05-Dir (**a**). Agarose gels showing full-genome PCR products, gel-purified 12 kb PCR products, and PCR-amplified linear minimal cloning vector pHaHdmin (**b**). The effectiveness of LLHR in the presence of arabinose was quantified by colony counting after selection on ampicillin containing LB-plates (LB amp+) (**c**). The mean of absolute colony numbers from three independent recombination experiments are shown with indicated standard deviation by error bars (**d**). Figure and legend reproduced from Nolden et al., 2016 by Creative Commons Attribution 4.0 International License [5]

at its 5′-end. The 3′-end of the olionucleotide hybridizes to the Hepatidis Delta Virus ribozyme sequence (Hd) in pHaHdmin (5′-GGGTCGGCATGGCATCTCCAC-3′).

2. Set up a 50 µl PCR reaction mixture consisting of 35.5 µl dH_2O, 10 µl 5× GoTaq PCR reaction buffer (containing 7.5 mM

$MgCl_2$), 1.0 μl dNTP (10 mM each), 1.0 μl of each upstream and downstream oligonucleotide (10 mM each), 1.0 μl pHaHd-min plasmid (100 ng/μl), 0.5 μl GoTaq polymerase (5 U/μl).

3. After 2 min initial denaturation, perform 30 cycles of PCR amplification with the following temperature profile: 1 min denaturation at 95 °C, 1 min annealing at 57–62 °C, 2.5 min elongation at 72 °C. The optimal annealing temperature may vary depending on the sequences of used oligonucleotides. Incubate another 5 min at 72 °C for final extension.
4. After PCR amplification, add 1 μl *Dpn*I directly to the PCR reaction to destroy methylated template DNA.
5. Incubate for 1 h at 37 °C.
6. Check the 2.7 kb PCR product by standard agarose gel electrophoresis and purify the linear vector DNA fragment from gel.

3.5 Recombinatorial Virus cDNA Cloning by LLHR

Recombination of linear vector DNA fragment with 12 kb full virus genome cDNA fragments is performed by co-electroporation of both DNA fragments into *E. coli* strain GB05-dir and subsequent induction of RecE/T recombinases by arabinose (Fig. 3a). After plating of electroporated *E. coli* successful recombination is indicated by appearance of multiple ampicillin-resistant clones. The integration of the complete virus genome cDNA into the plasmids is verified by plasmid purification from *E. coli*, downstream restriction endonuclease digests and DNA sequencing by standard methods

1. Inoculate 1.0 ml LB medium without antibiotics with *E. coli* GB05-Dir and incubate the culture overnight at 37 °C with shaking at 1100 rpm.
2. Before starting the next day, chill distilled H_2O and MicroPulser™ Cuvettes on ice for at least 2 h. Cool benchtop centrifuge to 2 °C.
3. For a single LLHR recombination reaction set up two 1.5 ml reaction tubes with 1.4 ml LB medium and inoculate with 30 μl of the *E. coli* GB05 overnight culture. Use vent caps or puncture the lid of the tube in order to guarantee air exchange. For a control reaction provided with the *E. coli* strain set up another two 1.5 ml reaction tubes with 1.4 ml LB medium and inoculate with 30 μl overnight culture. Incubate the tubes at 37 °C for 1.5 h shaking at 1100 rpm until OD600 ~0.3.
4. For induction of the RecE/T recombination proteins, add 50 μl 10% (w/v) L-arabinose to one of the tubes for the RABV cDNA recombination and to one of the control tubes, giving a final concentration of 0.3–0.4%. Leave the other tubes without induction as negative controls. Incubate all at 37 °C, shaking for 35 min (*see* **Note 2**).

5. Spin down *E. coli* cultures for 30 s in the 2 °C cooled benchtop centrifuge at 11,000 rpm (at 2 °C). Discard the supernatant by quickly tipping it out twice, and place the pellet on ice.
6. Resuspend the pellet with 1 ml chilled H_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again.
7. Centrifuge and tip out the supernatant once more; 20–30 µl will be left in the tube with the pellet. Keep the tubes on ice.
8. Add 100 ng of the RABV cDNA and 100 ng of the linear pHaHdmin plasmid backbone to each of the two microfuge tubes (induced and non-induced), and pipette the mixture into the chilled electroporation cuvette. In parallel, pipette 1 µl control vector and 1 µl insert provided with the GB05-dir *E. coli* into each of the two tubes of the control.
9. Electroporate at 1350 kV (*see* **Note 3**).
10. Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37 °C with shaking for about 90 min. Recombination will now occur.
11. Spin down the cells, remove 600 µl of the supernatant and resuspend the *E. coli* cells in the remaining 400 µl LB media. Plate the cells onto LB agar plates containing 100 µg/ml ampicillin and incubate overnight at 37 °C (*see* **Note 4**).
12. To confirm the correct recombination event, pick 10–20 colonies from the LLHR experiment and 2 from the control reaction, isolate plasmid DNA, and analyze the DNA by restriction digestion (*see* **Note 5**).

3.6 Recovery of Infectious Recombinant RABV from Cloned Plasmid DNA

Infectious recombinant virus is recovered from the cloned plasmids by co-transfection of the full-length cDNA plasmid with expression plasmids for RABV N, P, and L as described elsewhere [3–5]. Importantly, expression plasmids for attenuated virus SAD L16 have been shown to support also rescue of other RABVs, inclusive field isolates such as dog- and fox-derived RABVs [5]. It is thus not required to clone individual N, P, and L expression plasmid for each new isolated targeted by LLHR cloning (*see* **Note 6**).

4 Notes

1. Downstream of the ligation site the sequence quality may drop off, sequencing from both sites is recommended for correct end-sequence determination.
2. Do not use D-arabinose. Prepare a 10% L-arabinose stock solution (in distilled H_2O), and use it fresh or frozen in small aliquots at −20 °C.

3. A 5 ms pulse is recommended.
4. Although most ampicillin-resistant colonies will contain the correct plasmid recombinant, it is possible that secondary recombination, usually deletions between internal repeats in the plasmid, can also occur.
5. To confirm correct full-genome recombination of the RABV cDNA from fox and dog isolates into pHaHdmin, we used a SacI restriction digest (Fig. 4a), but any other restriction digest can be used. We recommended to use restriction endonucleases that cuts a minimum of three times and a maximum of eight times within the vector construct in order to confirm the correct size of the inserted cDNA genome.
6. Although recovery of infections recombinant RABVs will be successful in more than 50% of the cloned RABV cDNA plasmids, we recommend sequencing to exclude obvious mutations that negatively affect recovery of infections virions.

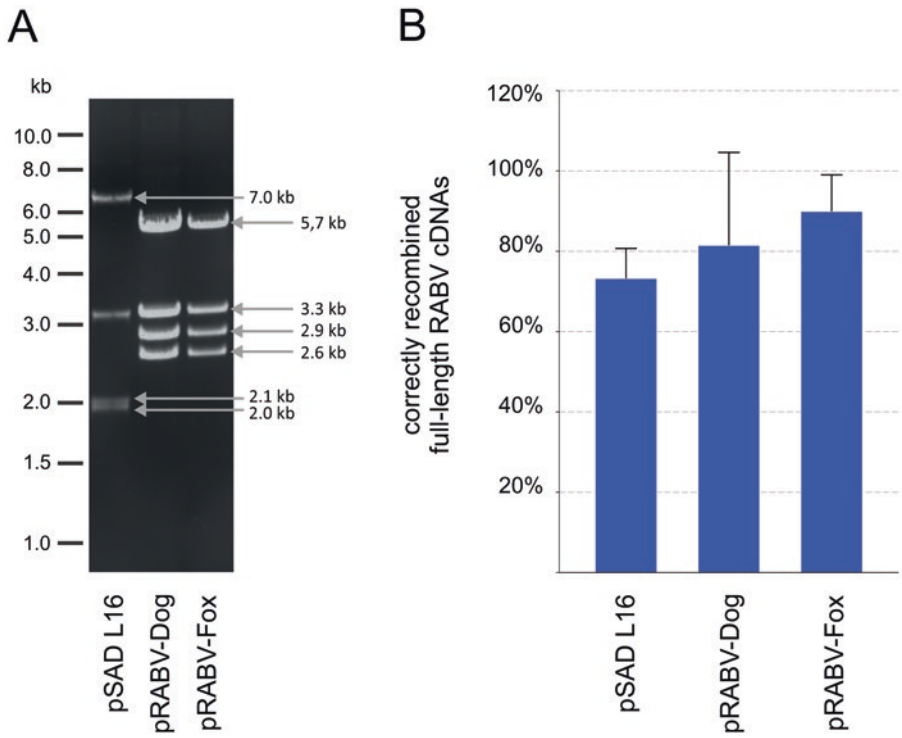

Fig. 4 SacI digest of ampicillin-resistant recombinants revealed that LLHR of full-length RABV cDNAs occurs at high frequency. (**a**) Exemplary restriction pattern of pSADL16, pRABV-Dog, and pRABV-Fox full-length cDNA plasmids after SacI digest. A virus cDNA clone was considered positive, when the overall fragment size corresponded approximately to 11.9 kb virus genome plus 2.6 kb vector size. (**b**) Correctly recombined full-length RABV genomes were averaged from two independent LLHR experiments and expressed as percentage of analyzed recombinants. Figure and legend reproduced from Nolden et al., 2016 by Creative Commons Attribution 4.0 International License [5]

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Chapter 13

Lassa Virus Reverse Genetics

Luis Martínez-Sobrido, Slobodan Paessler, and Juan Carlos de la Torre

Abstract

The Old World (OW) arenavirus Lassa (LASV) is estimated to infect several hundred thousand people yearly in West Africa, resulting in high numbers of Lassa fever (LF), a viral hemorrhagic fever (HF) disease associated with high morbidity and mortality. To date, no licensed vaccines are available to LASV infections, and anti-LASV drug therapy is limited to an off-label use of ribavirin (Rib) that is only partially effective. The development of reverse genetics has provided investigators with a novel and powerful approach for the investigation of the molecular, cell biology, and pathogenesis of LASV. The use of cell-based LASV minigenome (MG) systems has allowed examining the *cis*- and *trans*-acting factors involved in genome replication and gene transcription and the identification of novel drugable LASV targets. Likewise, it is now feasible to rescue infectious recombinant (r)LASV entirely from cloned cDNAs containing predetermined mutations in their genomes to investigate virus-host interactions and mechanisms of pathogenesis, as well as to facilitate screens to identify antiviral drugs against LASV and the implementation of novel strategies to develop live-attenuated vaccines (LAV). In this chapter we will summarize the state-of-the-art experimental procedures for implementation of LASV reverse genetics. In addition, we will briefly discuss some significant translational research developments that have been made possible upon the development of LASV reverse genetics.

Key words Recombinant Lassa virus, Lassa virus reverse genetics, Lassa virus rescue systems, Lassa virus minigenome assays

1 Introduction

The family *Arenaviridae* consists currently of two genera: (1) Mammarenavirus, with more than 25 recognized virus species that are classified into two distinct groups, Old World (OW) and New World (NW) arenaviruses [1], and (2) Reptarenavirus, a new genus that has been established to accommodate the recently discovered snake arenaviruses [2]. Genetically, OW arenaviruses constitute a single lineage, while NW arenaviruses segregate into clades A, B, A/B, and C [1]. Mammarenaviruses cause chronic infections in rodents with a worldwide distribution [1]. Asymptomatically infected animals move freely in their natural habitat and may invade human dwellings. Humans are infected most likely through

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mucosal exposure to aerosols, or by direct contact between infectious materials and abrade skin [1]. Several arenaviruses, chiefly Lassa (LASV) in West Africa [1] and Junin (JUNV) in the Argentinean Pampas [3], cause hemorrhagic fever (HF) disease in humans and represent important public health problems in their endemic areas. Moreover, evidence indicates that the worldwide-distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical significance [4] that poses a special threat to immunocompromised individuals [5]. In addition, several arenaviruses including LASV have features that make them credible biodefense threats [6].

LASV is the arenavirus with the highest impact in public health due to its large endemic region in West Africa and size of the population at risk of infection [7]. LASV is estimated to infect several hundred thousand individuals yearly in West Africa resulting in a high number of Lassa fever (LF) cases associated with high morbidity and significant mortality [8]. Recent studies indicate that LASV endemic regions are expanding [9] and the recent identification of Lujo virus (LUJV) [10], an OW arenavirus associated with an outbreak of HF in Southern Africa in 2008, have raised concerns about the emergence of novel HF OW arenaviruses outside their current known endemic regions. The estimated global burden of LF is high, and increased travel has led to the importation of LF cases into the United States, Europe, Japan, and Canada [11]. Concerns about human pathogenic arenavirus infections are aggravated by the lack of Food and Drug Administration (FDA)-licensed vaccines and existing antiviral drug treatment being limited to the use of ribavirin (Rib) that is only partially effective [12].

Arenaviruses are enveloped viruses with a bi-segmented negative-sense, single-stranded, RNA genome [1]. Each arenaviral segment encodes, using an ambisense coding strategy, two viral proteins in opposite orientation separated by a noncoding intergenic region (IGR) between the two viral genes (Fig. 1a) [1]. The large (L) segment encodes the viral RdRp or L polymerase protein (Fig. 1a, blue) involved in viral replication and gene transcription [13], and, in opposite direction, the small really interesting new gene (RING) finger protein Z (Fig. 1a, orange) that is the counterpart of the matrix (M) protein present in other negative-stranded (NS) RNA viruses and the major driving force of arenavirus budding and assembly [14]. The small (S) segment encodes for the viral GP (Fig. 1a, purple) that is post-translationally cleaved to form the two mature virions glycoproteins (GP1 and GP2) involved in receptor binding, cell entry and fusion [1]; and the viral nucleoprotein (NP) (Fig. 1a, green), which encapsidates the viral RNA and, together with the polymerase L and the viral RNA form the viral ribonucleoproteins (vRNPs) that are the minimal factors involved in arenavirus genome replication and gene transcription [13, 15]. In addition, arenavirus NP mediates the incorporation of

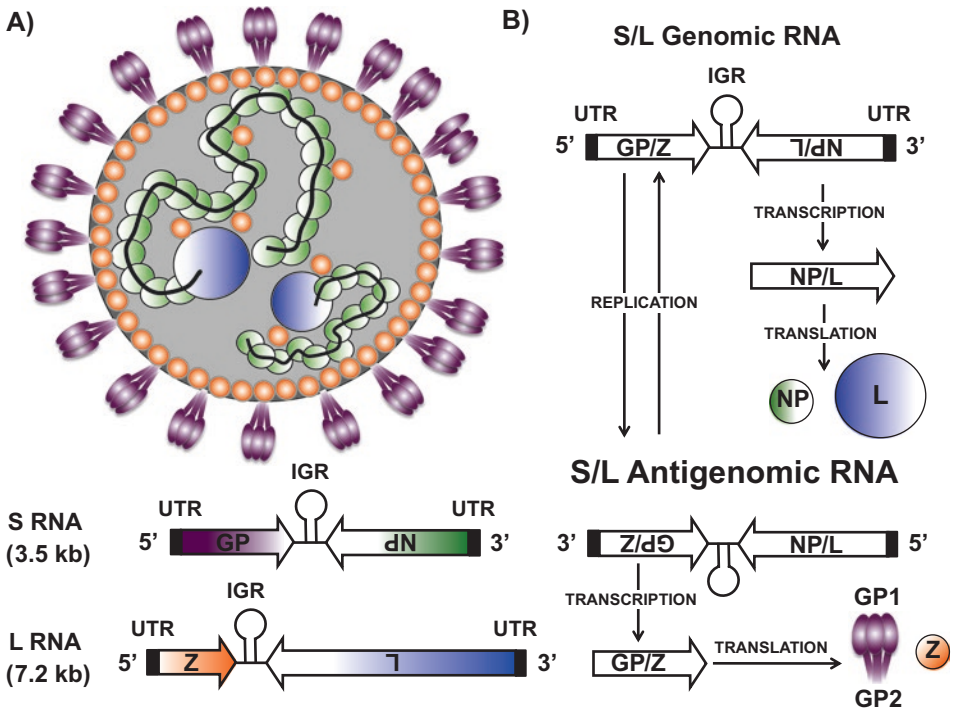

Fig. 1 Lassa virion structure, genome replication, and gene transcription. (**a**) Virion structure (**top**): Lassa viruses (LASV) are surrounded by a lipid bilayer containing the post-translationally processed viral glycoprotein precursor (GP, **purple**) involved in receptor binding (GP1) and cell entry (GP2). Underneath the viral membrane is a layer composed of the Z protein (**orange**), which plays a major role in viral assembly and budding, and is the counterpart of the matrix protein present in other enveloped negative-stranded RNA viruses. The core of the virus is made of two viral ribonucleoprotein (vRNP) complexes, composed of the viral genome segments (**black lines**) encapsidated by the viral nucleoprotein (NP, **green**). NP-Z interaction mediates incorporation of the vRNPs into LASV virions. Associated with the two vRNPs is the viral RNA-dependent RNA polymerase protein (L, **blue**) that, together with NP, are the minimal components required for LASV genome replication and gene transcription. Genome organization (**bottom**): LASV contain a single-stranded bi-segmented RNA genome of negative polarity. Each of the two viral (v)RNA genome segments uses an ambisense coding strategy to direct the synthesis of two viral proteins in opposite orientation. The Small (S) RNA segment (3.5 kb) encodes the viral GP and NP. The Large (L) RNA segment (7.2 kb) encodes the viral L and Z. (**b**) LASV genome replication and gene transcription: The L polymerase associated with the vRNPs initiates transcription from the viral promoter located within the untranslated region (UTR, **black boxes**) at the 3′ termini of the vRNAs. Primary transcription results in the synthesis of NP and L mRNAs from the S and L segments, respectively. Transcription termination is mediated by the intergenic region (IGR), a secondary stem-loop structured found in both vRNA segments between each of the two viral genes. Subsequently, the virus polymerase L adopts a replicase form and moves across the IGR to generate a copy of the full-length antigenome S and L vRNAs. The S and L antigenomic RNA segments serve as templates for the synthesis of GP and Z mRNAs, respectively. The antigenomic S and L vRNA segments serve also as templates for the amplification of the corresponding viral RNA genome species. LASV cycle takes place entirely in the cytoplasm of infected cells

the vRNPs into mature infectious virions by interacting with Z [16]. Furthermore, NP has also been shown to counteract the cellular host type-I interferon (IFN-I) [17–22] and inflammatory [21, 23] responses during viral infection.

Arenaviruses replication cycle takes place entirely in the cytoplasm of infected cells [1]. Homo-trimer complexes, consisting of the GP1 globular head and GP2 stalk region, form the spikes that decorate the surface of the arenavirus envelope [1] (Fig. 1a). GP1, located at the top of the spike, mediates attachment of the virus particle to receptors located in the surface of the cell [1]. Alpha-dystroglycan (αDG) has been described as the main receptor for OW and NW clade C arenaviruses [24]. However, clade A, B, and A/B NW arenaviruses appear to use the human transferrin receptor I as the cellular receptor for viral entry [25]. Upon viral attachment, arenavirus enter the cell via receptor-mediated endocytosis [1]. Fusion of the virion and the endosome membranes is triggered by acidification of the endosome, which induces a conformational change in the viral GP1/GP2 complex [1]. Fusion of the viral and cellular membranes releases the vRNPs into the cytoplasm of infected cells, where viral RNA replication and gene transcription occur [1].

Arenavirus gene transcription is mediated by the viral promoters located within the untranslated regions (UTRs) at the 3′ termini of viral RNA (vRNA) and complementary RNA species (cRNA) [1] (Fig. 2a). NP and L proteins, located at the 3′-end of the S and L viral segments, respectively, are translated from mRNAs with

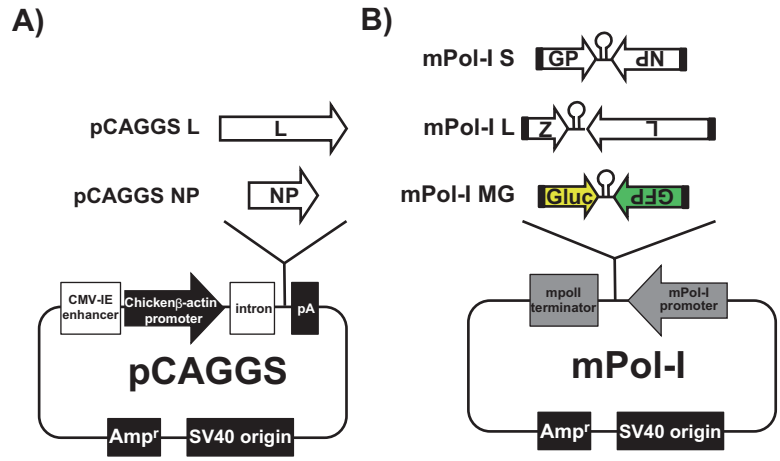

Fig. 2 Schematic representation of the plasmids used for LASV reverse genetics: (**a**) pCAGGS protein expression plasmids: Protein expression pCAGGS plasmids use the chicken β-actin promoter (**black arrow**) and the rabbit b-globin polyadenylation (pA) signal (**black box**) sequences to direct the synthesis of LASV L (**top**, pCAGGS L) and NP (**bottom**, pCAGGS NP). These plasmids are required to provide the minimal proteins required to initiate LASV gene transcription and genome replication. (**b**) Mouse polymerase I-driven vRNA expression plasmids: vRNA expression plasmids under the control of the mouse polymerase I (mPol-I) promoter (**gray arrow**) and terminator (**gray box**) sequences direct the synthesis of LASV vRNA S (**top**) and L (**middle**) segments. For the minigenome (MG) assays, a mPol-I vcRNA plasmid S where reporter genes substitute the viral NP (GFP, **green**) and GP (Gluc, **yellow**) is used (**bottom**), mPol-I MG

antigenomic sense polarity transcribed directly from the vRNAs and, therefore, are the first arenaviral proteins encoded upon infection [1] (Fig. 2b). The 3′-ends of the non-polyadenylated viral mRNAs have been mapped to a predicted a stem-loop structure within the noncoding intergenic region (IGR) found between the two viral genes in each vRNA segment [1] (Fig. 2b). GP and Z proteins, on the other hand, are located, respectively, at the 5′-end of the S and L viral segments and are not translated directly from mRNA derived from the vRNAs but from antigenome complementary RNA species after replication of the vRNAs [1] (Fig. 2b). Complementary RNA segments also serve as templates for the synthesis of nascent vRNAs [1] (Fig. 2b). Newly synthesized vRNAs are encapsidated by the viral NP to form the vRNP complexes and are packaged into progeny infectious virions by interaction of the viral Z [16]. Arenavirus assembly involves the interaction of the new vRNP complexes with the GP1/GP2 complexes present in the membrane of infected cells, a process mediated by interaction with the Z protein [26].

The development of arenavirus reverse genetics systems has provided investigators with new and powerful experimental approaches for the investigation of the *cis*-acting and the *trans*-acting factors that control the replication cycle of arenaviruses, including viral cell entry, genome viral replication and gene transcription, assembly and budding [16, 27]. Reverse techniques have also allowed the generation of recombinant arenaviruses with mutations in their genomes to examine their contribution in viral replication using cell cultures as well as in viral pathogenesis and associated disease using validated animal models of infection [18, 28]. Likewise, implementation of arenavirus reverse genetics have allowed researchers to study arenavirus-host interactions [18–20, 27, 29, 30], and potentiated the generation of novel candidate live-attenuated arenavirus vaccines [29–34] as well as the development of screening methods to identify and evaluate novel anti-arenavirus drugs targeting specific steps of the virus life-cycle [29, 32, 35]. Reverse genetics of arenaviruses have also been used for the generation of recombinant tri-segmented (r3) arenavirus expressing additional genes of interest (GOI) [29, 30, 33, 34] to facilitate the study of several members in the family, including the identification of antivirals inhibiting the replication cycle of arenaviruses [35] as well as their possible implementation as vaccine vector candidates [29, 30, 34]. Finally, arenavirus reverse genetics have been used for the generation of a single-cycle infectious, reporter-expressing, recombinant arenaviruses that are limited to replicate in GP-expressing complementing cell lines [32, 36], allowing the study of some aspects of the biology of highly pathogenic arenaviruses (e.g., neutralizing antiviral responses and identification of inhibitors of GP-mediated cell entry) without the use of special biosafety level (BSL) laboratory conditions [32]. In this chapter we will focus on reverse genetics techniques, based

on BHK-21 cells, for LASV that requires BSL4 conditions, with appropriate safety and security measures (*see* Subheading 3.4).

2 Materials

2.1 LASV Reverse Genetics Plasmids

We have developed an RNA mPol-I/Pol-II reverse genetics system for the rescue of a molecular clone of the highly pathogenic Josiah strain of LASV lineage IV [37]. Plasmids for the generation of recombinant LASV (rLASV) (Fig. 2) can be grown in DH5α-competent bacteria (Invitrogen) using Luria broth (LB) media in the presence of 100 μg/mL of Ampicillin (Fisher Scientific) at 37 °C for 16–18 h, with the exception of the mPol-I L plasmid (*see* **Notes 1** and **2**) [37]. Plasmids can be purified using commercial maxiprep kits (e.g., Qiagen) following manufacturer's recommendations and stored at −20 °C (*see* **Notes 3** and **4**).

1. pCAGGS *trans*-acting factors protein expression plasmids: pCAGGS plasmid contains the chicken polymerase II (Pol-II)-driven β-actin promoter and the rabbit β-globin polyadenylation (pA) signal sequence to produce LASV NP and L, which are the minimum components for LASV replication and transcription (Fig. 2a) required to evaluate LASV genome replication and gene transcription using the minigenome (MG) approach (Fig. 3) and for the generation of recombinant rLASV (Fig. 4) [37]. A pCAGGS plasmid encoding ostracod *Cypridina noctiluca* luciferase (Cluc) secreted luciferase is used in the MG assay (Fig. 3) to normalize transfection efficiencies [29, 31, 34, 35] (*see* **Notes 5** and **6**).
2. mPol-I vRNA expression plasmids: LASV reverse genetics use the mouse RNA polymerase I (mPol-I) promoter to direct intracellular synthesis of S and L genome or antigenome, RNA species [37], that is species specificity [29]; and the mPol-I terminator to obtain authentic LASV vRNA 3′-ends [37] (Fig. 2b) (*see* **Notes 2** and **7**). LASV RNA replication and gene transcription can be evaluated using a LASV vRNA-like MG plasmid (mPol-I MG) (Fig. 2b) [15, 16, 28–31, 34, 35] where LASV GP and NP open reading frames (ORFs) are replaced by reporter genes. In our case, we replaced GP for *Gaussia* luciferase (Gluc) and NP for the green fluorescent protein (GFP) [15, 16, 28–31, 34, 35, 37] (Fig. 3) (*see* **Notes 8** and **9**). For the generation of rLASV (Fig. 4), two mPol-I plasmids expressing the S (mPol-I S) and L (mPol-I L) viral genomic or antigenomic RNAs are used [37] (Fig. 2b) (*see* **Note 2**).

2.2 Cell Lines for LASV MG Assays and for the Generation of rLASV

The experimental procedures used for the development of LASV MG systems and rescue of infectious rLASV described here are based on the use of the mPol-I promoter to direct intracellular synthesis of the appropriate MG vRNA-like (Figs. 2b and 3), or

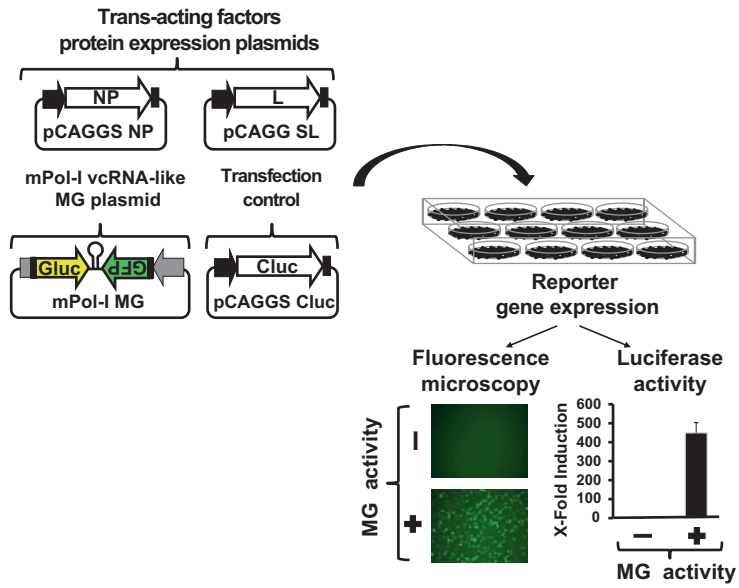

Fig. 3 LASV minigenome (MG) assay: To assess LASV genome replication and gene transcription using a MG approach, susceptible mouse cells (6- or 12-well plate, triplicates) are transiently co-transfected, using LPF2000, with the pCAGGS plasmids driving the expression of LASV NP and L, and the mPol-I vcRNA S-like plasmid where reporter genes substitute for the viral NP (GFP, **green**) and GP (Gluc, **yellow**), together with a pCAGGS plasmid expressing **Cypridina noctiluca** (Cluc) to normalize transfection efficiencies. Recommended amounts of **trans**-acting factors protein expression pCAGGS and mPol-I vcRNA-like MG plasmids, LPF2000 and cells for LASV MG assays in 12-well or 6-well plates are detailed in Table 1. Reporter gene expression is then evaluated by GFP expression using a fluorescent microscope (**left**) and by Gluc activity using a luminometer (**right**). Reporter Gluc expression is represented as fold induction over cells transfected with an empty pCAGGS plasmid instead of LASV NP (negative control, –). **Black arrows and boxes** indicate the chicken b-actin promoter and the rabbit b-globin polyadenylation (pA) signal in the pCAGGS protein expression plasmids. LASV untranslated (UTR, **black boxes)** and intergenic (IGR) regions are indicated in the mPol-I vRNA-like MG plasmid. The mPol-I promoter (**gray arrow**) and terminator (**gray box**) sequences in the mPol-I vRNA-like MG plasmid are also indicated

recombinant S and L genome or antigenome RNA species (Figs. 2b and 4), respectively [37] (*see* **Note 7**). The activity of the mPol-I promoter exhibits species specificity and therefore is restricted to rodent cells [29]. We use baby hamster kidney (BHK-21) cells since they are easy to maintain, have high transfection efficiencies, and are able to produce high viral titers [29, 30, 33, 34]. BHK-21 cells are available from the American Type Culture Collection (ATCC, catalogue number CRL-1596). Vero (African green monkey kidney epithelial) cells (LASV titration) are available from the ATCC (catalogue number CRL-1596). Vero cells are used to amplify rLASV from tissue culture supernatants collected from

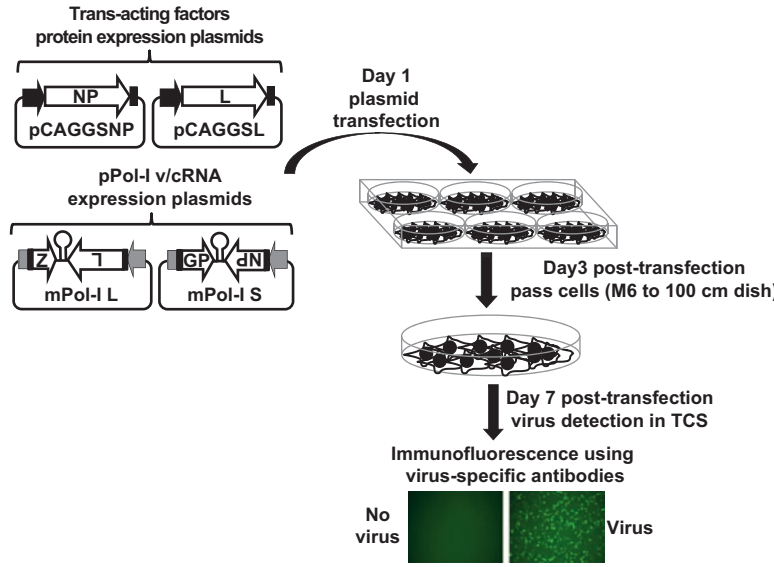

Fig. 4 Generation of rLASV: LASV rescues are performed using rodent BHK-21 cells in 6-well plate format (triplicates). Cells are transiently co-transfected, using LPF2000, with the pCAGGS protein expression plasmids encoding LASV NP and polymerase L (required to initiate viral gene transcription and genome replication) together with the mPol-I vRNA expression plasmids encoding the viral S and L segments (required to provide LASV vRNAs to initiate viral gene transcription and genome replication). Recommended mounts of pCAGGS and mPol-I plasmids, LPF2000 and BHK-21 cells for LASV wild-type (WT) rescues are indicated in Table 2. At 72 h post-transfection, BHK-21 cells are trypsinized and scaled-up into 10 cm dishes. After an additional 96 h incubation period, tissue culture supernatants (TCS) are collected and presence of LASV is determined by immunofluorescence using LASV specific antibodies. The chicken b-actin promoter (**black arrow**) and the rabbit b-globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated (UTR, **black boxes**) and intergenic (IGR) regions in the mPol-I vRNA expression plasmids are indicated. The mPol-I promoter and terminator sequences in the mPol-I plasmids are indicated by **gray arrows** and **boxes**, respectively

transfected BHK-21 cells [37]. Both cell lines are maintained in T-75 flasks at 37 °C in a 5% CO_2 atmosphere with DMEM 10% FBS, 10% FBS, 1% PS.

2.3 Tissue Culture Media and Reagents

1. DMEM 10% FBS, 1% PS: This media is used for maintenance of both BHK-21 and Vero cells. Mix 445 mL Dulbecco's modified Eagle's medium (DMEM, Invitrogen), 50 mL of Fetal Bovine Serum (FBS, Atlanta Biologics), and 5 mL of 100× Penicillin/Streptomycin (PS, Invitrogen). Store at 4 °C.
2. Opti-MEM I medium.
3. Infection Media: Mix (2:1) Opti-MEM I and DMEM 10% FBS 1% PS. Store at 4 °C (*see* **Note 10**).

4. 10× PBS (Phosphate buffered saline): Mix 80 g of NaCl, 2 g of KCl, 11.5 g of $Na_2HPO_4•7H_2O$, 2 g of KH_2PO_4. Add ddH_2O up to 1 L. Adjust pH to 7.3 and sterilize by autoclaving. 10× PBS can be stored at room temperature (room temperature). Working concentration (1× PBS) should be prepared by diluting (1:10) 10× PBS with ddH_2O. Sterilize by autoclaving. Store at room temperature.
5. Bovine serum albumin (BSA) 2.5%: 2.5 g of BSA (Sigma) in 97.5 mL of 1× PBS. Store at 4 °C.
6. Lipofectamine 2000 (LPF2000): A LPF2000:DNA ratio of 2.5:1 for both LASV MG (Table 1) and virus rescue (Table 2) is recommended.

Table 1
Plasmid DNA and LPF2000 concentrations for LASV minigenome (MG) assays

Plasmid	6-well plates (μg)	12-well plates (μg)
pCAGGS NP	0.6	0.3
pCAGGS L	1.2	0.6
mPol-I S MG	1.0	0.5
pCAGGS Cluc	0.2	0.1
Total DNA	3.0	1.5
LPF2000	7.5	3.75
Cells	~1.0–1.2 × 10^6/well	~5.0–6.0 × 10^5/well

Recommended amounts of pCAGGS and mPol-I plasmids, and LPF2000 required to assess viral gene replication and genome transcription using a LASV MG assay in 12-well (5.0–6.0 × 10^5 cells/well, left) or 6-well (1.0–1.2 × 10^6 cells/well, right) plates are indicated

Table 2
Amount of plasmid DNA, LPF2000, and BHK-21 cells for LASV rescues

Plasmid	6-well plates (μg)
pCAGGS NP	0.8
pCAGGS L	1.0
mPol-I S	0.8
mPol-I L	1.4
Total DNA	4.0
LPF2000	10
Cells	~1.0–1.2 × 10^6/well

Recommended amounts of pCAGGS and mPol-I plasmids and LPF2000 to generate recombinant wild-type (WT) LASV in 6-well plates (1.0–1.2 × 10^6 cells/well) are indicated

7. Trypsin-EDTA.
8. Luciferase Assay kits: Biolux Gaussia and Cypridinia Luciferase Assay kits from New England Biolabs using a Lumicount luminometer (Packard).
9. Plasticware for Tissue culture: 6, 12, and 96 wells cell culture plates and 10 cm tissue culture dishes (Greiner Bio-one).

3 Methods

3.1 LASV Minigenome (MG) Systems to Evaluate Viral Genome Replication and Gene Transcription

For LASV MG assays, BHK-21 cells are co-transfected with the mPol-I-driven plasmid regulating expression of a reporter-expressing vRNA-like segment (e.g., Gluc and GFP) together with Pol-II-driven protein expression plasmids encoding NP and L [13], and a Pol-II-dependent Cluc to normalize transfection efficiencies (Figs. 2 and 3) (*see* **Notes 8**, **11** and **12**). Efficient recognition of the LASV-like reporter-expressing RNA by the viral NP and L requires precise 3′ and 5′-ends in the vRNA [29, 38]. In the case of the mPol-I plasmid, efficient vRNA 5′-ends are obtained with the use of the mPol-I promoter [29, 38]. To obtain authentic LASV vRNA 3′-ends, either the self-cleaving hepatitis delta virus ribozyme (HDVrz) or the mPol-I terminator (T) sequences can be used [29] (*see* **Notes 13** and **14**).

1. LASV MG transfection mix (Fig. 3):
   (a) Opti-MEM I-Lipofectamine 2000 (LPF2000) mix for one well in a six-well plate. In a 1.5 mL microcentrifuge tube mix

Opti-MEM I	250 μL
LPF2000	7.5 μg
Incubate 5–10 min at room temperature	

   LASV MG assays can be performed using a 6- or 12-well plate format. Adjust amount of plasmid DNA, LPF2000, and cells accordingly (Table 1, *see* **Notes 15** and **16**).
   (b) Opti-MEM I-DNA plasmid mix. In a separate 1.5 mL tube, prepare the LASV MG DNA plasmid mixture using the recommended amounts (Table 1) in 50 μL of Opti-MEM I media.
   (c) Combine the Opti-MEM I-LPF2000 mix by adding it into the tube containing the Opti-MEM I-DNA plasmid mix.
   (d) Incubate Opti-MEM I-LPF2000-DNA plasmid mixture for approximately 20–30 min at room temperature. During this incubation period, prepare the cells for transient transfection in suspension (*see* **Note 17**).

2. Preparation of BHK-21 cells (*see* **Notes 18** and **19**): Prior to cell manipulation bring the 1× PBS, DMEM 10% FBS, 1% PS media, and trypsin-EDTA mixture to 37 °C for approximately 10 min.
   (a) Wash the cells, twice, with 5 mL 1× PBS.
   (b) Trypsinize the cells using 1 mL trypsin-EDTA. BHK-21 detachment is usually accomplished by incubating the cells in a 37 °C, 5% CO_2 humidified incubator for approximately 5 min.
   (c) Gently tap the tissue culture plate to expedite this process and to confirm all the BHK-21 cells have been detached.
   (d) Once the cells have been fully detached, resuspend them in 9 mL of DMEM 10% FBS, 1% PS.
   (e) Transfer the BHK-21 cells to a 15 mL tube and centrifuge for 5 min at 1000 × *g*.
   (f) Remove the media and resuspend the cells in 10 mL of fresh DMEM 10% FBS, 1% PS.
   (g) Count cells using a hemocytometer and adjust the cell concentration to 1.0 × 410^6 cells/mL. Successful LASV MG assays are typically achieved by transfecting approximately 1–2 × 10^6 (for 6-well plates) or 5–6 × 10^5 (for 12-well plates) cells per transfection (Table 1).
3. LPF2000/DNA transfection into BHK-21 cells:
   (a) To Opti-MEM I-LPF2000-DNA plasmid mix pipette into each LPF2000/DNA tube (**step 3**) 1 mL containing the right amount of BHK-21 cells (**step 4**) and let the LPF2000/DNA/cell mixture incubate for approximately 5 min at room temperature.
   (b) LPF2000/DNA/cells plating: Transfer the LPF2000/DNA/cell mixture (**step 5**) into individual 6- or 12-well tissue culture plates. Gently tap the plates to uniformly distribute the cells and incubate them in a 5% CO_2 humidified 37 °C incubator for approximately 6–12 h. Remove the tissue culture supernatant and replaced with 2 mL (6-well plates) or 1 mL (12-well plates) of fresh DMEM 10% FBS, 1% PS.
4. Measure LASV MG reporter gene activity: After incubating the transfected BHK-21 cells for a total of approximately 48 h, measure the MG reporter gene activity. Depending on the genes encoded by the MG plasmid, the assay can be determined qualitatively by fluorescence microscopy (e.g., GFP expression) or quantitatively (e.g., Gluc) using a Lumicount luminometer (Packard) (*see* **Note 20**). Reporter gene activation (Gluc) is represented as fold induction over BHK-21 cells transfected with a negative pCAGGS empty plasmid control instead of LASV NP (negative control, −) (Fig. 3) [15, 16, 28–31, 34, 35].

3.2 LASV Rescue System Experimental Approach (Fig. 4)

1. Preparation of LPF2000/DNA transfection mix: First, mix 250 μL of Opti-MEM I media with 10 μg of LPF2000 (1:2.5 ratio of plasmid DNA/LPF2000) per transfection (Table 2). LASV rescues are performed in 6-well plates (*see* **Note 21**). Incubate the Opti-MEM I-LPF2000 mixture for approximately 5–10 min at room temperature. During this incubation time, prepare the Opti-MEM I-DNA plasmid mixture.
2. Opti-MEM I-DNA plasmid mixture: In a separate tube, prepare the LASV DNA plasmid rescue mix using the recommended amounts provided in Table 2 in a total volume of 50 μL of Opti-MEM I media.
3. Preparation of the LPF2000/DNA mixture: Combine Opti-MEM I-LPF2000 and Opti-MEM I-DNA plasmid by pipetting 250 μL of Opti-MEM I-LPF2000 (**step 1**) into the Opti-MEM I-DNA plasmid mixture (**step 2**) and incubate for approximately 20–30 min at room temperature. During this incubation period, prepare the cells for transient transfection in suspension (*see* **Note 17**).
4. Preparation of cells: Before manipulating the BHK-21 cells, bring the 1× PBS, DMEM 10% FBS, 1% PS media, and trypsin-EDTA mixture to 37 °C for approximately 10 min (*see* **Note 22**).
   (a) Wash the cells, twice, with 5 mL 1× PBS.
   (b) Trypsinize the cells using 1 mL trypsin-EDTA. BHK-21 detachment is usually accomplished by incubating the cells in a humidified 37 °C, 5% CO_2 chamber, for approximately 5 min. Gently tap the tissue culture plate to confirm all the BHK-21 cells have been detached.
   (c) After BHK-21 cells are completely detached from the plate, carefully resuspend them with 9 mL of DMEM 10% FBS, 1% PS. Place the total media/cell mixture in a 15 mL centrifuge tube and centrifuge the cells for 5 min at 1000 × *g*.
   (d) Remove the media and resuspend the BHK-21 cells in 10 mL of fresh DMEM 10% FBS, 1% PS. Count the cells using a hemocytometer and adjust the cell concentration to approximately 1.0–1.2 × 10^6 cells/mL (Table 2).
5. LPF2000/DNA incubation with BHK-21 cells: After approximately 20–30 min incubation, pipette into each LPF2000/DNA tube (**step 3**) 1 mL containing approximately 1.0–1.2 × 10^6 cells. Incubate the LPF2000/DNA/cell mixture for approximately 5 min at room temperature.
6. LPF2000/DNA/cells plating: Transfer the LPF2000/DNA/cell mixture (**step 5**) into individual wells of a 6-well tissue culture plate. Gently tap the plate to distribute the cells uniformly and incubate the cells in a 5% CO_2 humidified 37 °C

incubator for approximately 6–12 h. After 6–12 h incubation, replace the tissue culture supernatant and replace with 2 mL of infection media and return the cells to the incubator and incubate for 72 h [37].

7. Cell passage: After 3-days incubation, transfected BHK-21 cells should reach approximately 100% confluence. Remove the tissue culture supernatant, wash the cells twice with 1× PBS, and trypsinize them by adding 500 μL of trypsin-EDTA/well. Return the tissue 6-well plates to the incubator and let them incubate for approximately 5 min. Gently tap the plates to complete cell detachment from the plate. Carefully resuspend the BHK-21 cells with 1 mL of DMEM 10% FBS, 1% PS and transfer to a 1.5 mL microcentrifuge tube. Centrifuge the cells for 5 min at 5000 × *g*, 4 °C in a microcentrifuge, remove the tissue culture supernatant and resuspend the BHK-21 cells in 1 mL of infection media and transfer to a 10 cm tissue culture dish. Bring up the volume in the tissue culture dish to 10 mL with infection media. Gently shake the 10 cm dish to allow uniform distribution of the BHK-21 cells and incubate the cells at 37 °C, 5% CO_2 for an additional 96 h (*see* **Note 23**).
8. LASV recover from tissue culture supernatants: After 4 days of incubation, collect the tissue culture supernatants from the 10 cm tissue culture dishes (**step 7**) into a 15 mL centrifuge tube. Centrifuge at 2500 × *g*, 4 °C for 5 min. Collect the tissue culture supernatants containing LASV and discard the cell pellet. Aliquot the virus in cryotubes and store them at −80 °C (*see* **Note 24**). Aliquots can be stored at −80 °C until confirmation of the presence of LASV.

3.3 Confirmation of Successful LASV Rescue

Arenaviruses, including LASV, do not display classic cytopathic effect (CPE) observed with other NS RNA viruses [1]. Thus, successful rescue of LASV must be evaluated by immunofluorescence using arenavirus-specific antibodies [15, 16, 28–31, 34, 35] (*see* **Notes 25** and **26**).

1. A day before confirming LASV rescues or titrations, trypsinize Vero cells from 10 cm dishes as indicated above. In this case, adjust the cell density to 2 × 10^5 cells/mL. Seed the cells in 96-well plates (100 μL/well) and gently tap the plate so that a uniform cell monolayer of 80–90% confluence (~4 × 10^4 cells/well) is reached the next day. Incubate the plates at 37 °C, in the 5% CO_2 incubator.
2. On the day of LASV titration, serially diluted (tenfold dilutions) tissue culture supernatants recovered from transfected BHK-21 cells (or virus stocks) in Opti-MEM I.
3. Remove the media and wash Vero cells twice with 50 μL of 1× PBS. Infect the cells with 50 μL of the serially diluted tissue

culture supernatants. Allow virus adsorption at 37 °C, 5% CO_2, for 90 min, rocking the plates every 15 min to allow uniform virus infection of the Vero cell monolayers.

4. After 90 min infection, remove the tissue culture supernatants and add 100 μL of infectious media. Allow the cells to incubate for 16–18 h (*see* **Note 27**).
5. After 16–18 h, remove the tissue culture supernatants, fix the infected cells with 4% formaldehyde diluted in 1× PBS for 15 min at room temperature, before permeabilizing with 0.1% triton X-100 for 15 min at room temperature (*see* **Note 28**).
6. Remove the fixation/permeabilization solution and wash the cells, three times, with 50 μL of 1× PBS.
7. Block the cells with 2.5% BSA in 1× PBS for 1 h at room temperature. Alternatively, Vero cells can be blocked with 2.5% BSA ON at 4 °C.
8. During cell blocking, prepare the primary antibody. Antibodies specific to LASV antigens should be diluted in blocking solution (2.5% BSA), and centrifuge for 15 min at 3500 × *g* before use (*see* **Note 29**).
9. After blocking the cells, remove the blocking solution and incubate the cells with 50 μL of the primary LASV antibody. Incubate at 37 °C, 5% CO_2 for 1 h.
10. After incubation with the primary LASV antibody, wash the cells three times with 50 μL of 1× PBS. Incubate with 50 μL of the fluorescein-conjugated secondary antibody diluted (following manufacturer recommendations) in blocking solution (2.5% BSA) at 37 °C, 5% CO_2 for 30 min (*see* **Note 30**).
11. Following the 30 min incubation, remove the secondary antibody and wash the cells three times with 50 μL of 1× PBS.
12. At this moment, LASV rescue (or viral titers) can be determined under a fluorescent microscope. Viral titration is calculated by counting the fluorescent focus forming units (FFU) and the respective dilutions.

3.4 Amplification of LASV

Successful LASV rescue depends of multiple factors, including among others, the proper maintenance of BHK-21 cells, the quality of the plasmid preparations used for LASV rescues, and the transfection efficiency of BHK-21 cells. Thus, we recommend performing LASV rescues in triplicate in order to increase the likelihood of a successful rescue. If successful, it is possible that LASV titers in tissue culture supernatants from the initial rescue are low and, therefore, the virus need to be amplified to generate a stock. To that end, we recommend infecting fresh Vero cells at low multiplicity of infection, MOI (0.01) and allow LASV amplification for 48–72 h before collecting the new tissue culture supernatants for viral titration, as previously described.

3.5 Biosafety Conditions to Generate rLASV

Rescue of replication-competent rLASV must be done in BSL4 and following guidelines and standard operating procedures written in the corresponding BSL4 Standard Operating Procedures Manual that should meet the requirements outlined in 42 CFR 73 [37]. Samples that require further experimental steps outside of BSL4 should be either irradiated, fixed in formalin, or treated with TRIZOL reagent. Irradiation of samples containing infectious rLASV at 3 M Rads completely eliminate virus viability. All disposable materials leaving the BSL4 facilities are autoclaved, then placed in a biohazard container for incineration or non-disposable items are either autoclaved, fumigated, or chemically disinfected prior to their relocation. As documented for other BSL4 viral agents, including Ebola virus [39], the use of single-cycle infectious rLASV would overcome the need for BSL4 and speed up drug discovery process [32]. Multiplication of single-cycle infectious recombinant LASV where GFP substitutes for the virus GPC (rLASVΔGPC-GFP) would be confined to cells engineered to express the LASV GPC, which provides a safe way to handle LASV outside a BSL4 facilities to facilitate large-scale screening efforts to identify host cell factors required for LASV multiplication, as well as compounds with activity against LASV [32]. In support of this, experiments done with the prototypic arenavirus LCMV (BSL2) have provided compelling evidence that a recombinant arenavirus lacking its GPC gene is unable to propagate and cause disease in mice, the natural host of LCMV [36]. Thus, while intracranial (ic) inoculation of adult mice with 10^3 plaque-forming units (PFU) of WT rLCMC causes 100% lethality within 7 days, mice inoculated ic with up to 10^6 PFU of rLCMVΔGPC-GFP did not exhibit any clinical symptoms of disease [36]. Also, due to the nature of their genome it is extremely unlikely that any recombination could occur that would lead to generation of viable virus.

4 Notes

1. Because of its size, we recommend growing the mPol-I L plasmid at 30 °C for 24 h.
2. We have found that the orientation of the insertion dramatically influenced the genetic stability in bacteria of the mPol-I plasmid coding for the full-length L RNA. We recommend the construct containing the cDNA for the L RNA in a genome orientation with respect to the mPol-I promoter since the plasmid containing the L RNA in the opposite antigenomic orientation was significantly more prone to large deletions. It is worth noting to mention that both mPol-I L plasmid with either genomic or antigenomic orientation were successfully used to rescue rLASV with similar levels of efficiency [37].

3. Plasmid concentrations can be determined using spectrophotometry at 260 nm, with DNA purity being estimated using the 260:280 nm ratio (it is optimal to reach a 1.8–2.0 ratio for optimal LASV rescues).
4. All LASV pCAGGS and mPol-I plasmids can be generated using normal cloning techniques and sequenced using standard protocols.
5. We use a secreted luciferase, so both the MG Gluc and the transfection efficiency control Cluc can be measured directly from tissue culture supernatants [29, 31, 34, 35] (Fig. 3).
6. Other Pol-II-driven protein expression plasmids can be used instead of pCAGGS for LASV MG and rescue approaches. Likewise, other alternative reporter luciferases genes can be used for LASV MG assays and to normalize transfection efficiencies.
7. In this protocol we describe LASV reverse genetic approaches using the Pol-II/mPol-I system [37]. Alternatively, expression of vRNA S and L segments can be achieved with the use of T7-driven expression plasmids [27, 40]. In contrast to mPol-I, the T7 RNA polymerase promoter works similarly in different mammalian cells but its activity requires the incorporation into the transfection mix of an additional Pol-II-driven expression plasmid (e.g., pCAGGS) for the expression of the T7 RNA polymerase [27]. Since expression of the T7 RNA polymerase is based on plasmid transfection, any cell line can be used to evaluate LASV genome replication and gene transcription (MG assay, Fig. 3) or for viral rescues (Fig. 4). The Pol-II-driven T7 expressing plasmid should be added to the transfection mixtures described in Table 1 (MG) and Table 2 (LASV rescue). Alternatively, a cell line stably expressing the T7 RNA polymerase can be used to avoid the transfection of an additional plasmid. We recommend the use of BSR-T7 cells, a BHK-21 cell line constitutively expressing the T7 RNA polymerase [40].
8. Different reporter fluorescent proteins can be used instead of GFP to evaluate LASV MG activity under a fluorescent microscope. Likewise, an alternative no-secreted (e.g., Firefly or Renilla) or secreted (e.g., nano) luciferase can be used to evaluate LASV genome` replication and gene transcription using the MG assay (*see* **Note 5**).
9. Instead, LASV GP can be replaced by GFP and NP by Gluc [34]. It is important to state that reporter gene expression depends on its location in the LASV vRNA-like MG plasmid since it has been shown higher expression levels of NP than GP during arenaviral infections and MG transfections [34].

10. Alternatively, DMEM 2% FBS, 1% PS can be used during and after LASV infections.
11. Because of the species specificity of Pol-I promoters [29], LASV MG assays based on the mPol-I promoter should use a rodent cell line (e.g., BHK-21 cells).
12. Alternatively to the use of rodent cell lines, LASV MG assays can be performed in any cell line by using T7-driven polymerase promoters [27, 40]. In this case, transfection of a T7 polymerase expression plasmid should be added to the transfection mix, together with a T7-driven MG LASV plasmid (Table 1). Alternatively, a cell line constitutively expressing T7 RNA polymerase (e.g., BSR-T7 cells) can be used instead of a T7 polymerase expression plasmid [40].
13. We have found that the mPol-I T sequence is more efficient than the HDVrz sequence in producing arenavirus-like RNAs with the precise 3′-ends in both MG and virus rescue approaches [29].
14. With T7-driven polymerase plasmids, the precise 5′ and 3′-ends of both MG and virus rescue approaches are achieved by the use of the T7 promoter and terminator sequences, respectively [40].
15. We typically perform LASV MG assays in triplicate to get a good representation of the experiment and for statistical analysis.
16. Transfection in the absence of the pCAGGS plasmid encoding LASV NP should be included as a negative control (Fig. 3)
17. Alternatively, BHK-21 cells can be transiently transfected in monolayer. However, we have observed better transfection efficiencies for MG and viral rescues when BHK-21 cells are transfected in suspension.
18. We recommend transfecting rodent cells that are susceptible to LPF2000/DNA transfection, such as BHK-21 cells, to improve the quality and reproducibility of the MG experiments. Alternatively, other rodent cell line susceptible to DNA:LPF2000 transfection can be used for LASV MG assays.
19. LASV MG assays can be performed in other no-rodent cell lines by using the T7-driven polymerase promoters. In that case, in addition to a T7-driven LASV MG plasmid, transfection of a T7 polymerase expression plasmid should be added to the transfection mix (Table 1). Alternatively, a cell line constitutively expressing T7 RNA polymerase (e.g., BSR-T7 cells) can be used instead of a T7 polymerase expression plasmid [40].
20. The use of GFP and Gluc as reporter genes in the LASV MG assay allows the measurement of viral genome replication and gene transcription at different times post-transfection using the

same transfected cells [15, 16, 28–31]. We recommend assessing LASV MG activity first under a fluorescent microscope to have an estimation of LASV replication and transcription.

21. To increase the likelihood of successful LASV rescue, we recommend performing transfections in triplicate. Therefore, prepare enough Opti-MEM I-LPF2000 based on the number of LASV rescues planned.
22. LASV rescues can be performed in any rodent cell line because of the use of the mPol-I promoter [37]. We recommend the use of BHK-21 cells since they are easy to maintain, have high transfection efficiencies, and support LASV growth [29, 30, 33, 34]. Alternatively, LASV rescues can be performed in any cell type with the use of the T7-driven RNA polymerase plasmids [40]. In this case, add a T7 RNA polymerase expression plasmid into the transfection mix (Table 2). Alternatively, use cells constitutively expressing the T7 RNA polymerase (e.g., BSR-T7 cells) [40].
23. Alternatively, tissue culture supernatants collected from BHK-21 cells at 72 h post-transfection can be used to infect fresh monolayers of Vero cells to produce stocks with increased virus titers [37]. Usually, tissue culture supernatants collected form Vero cells at 72 h post-infection consistently had titers of approximately 10^6 focus forming units (FFU)/mL.
24. Make small volume aliquots to prevent multiple thaw cycles, which may reduce LASV titers.
25. We recommend the use of immunofluorescence assays (16–18 h) over plaque assays (5–6 days) to determine LASV titers [15, 16, 28–31, 34, 35].
26. We recommend performing LASV titrations in triplicates, using 96-well plates of Vero cells.
27. LASV infections over 18 h may lead to secondary infections and, therefore, result in overestimation of viral titers.
28. Alternatively, fix and permeabilize the cells with 4% formaldehyde, 0.1% triton X-100 diluted in 1× PBS for 15 min at room temperature.
29. An antibody specific to LASV NP is recommended since it is the most abundant viral protein produced during arenavirus infections and will assist in easy detection of LASV [1]. The species (e.g., mouse, rabbit), nature (e.g., monoclonal or polyclonal), and proper dilution of the antibody used for LASV detection and/or titration should be determined previously.
30. We normally use a secondary FITC-conjugated α-mouse antibody (Dako) diluted 1:100 in 2.5% BSA [15, 16, 28–31, 34, 35].

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Chapter 14

Reverse Genetics of Influenza B Viruses

Aitor Nogales, Daniel R. Perez, Jefferson Santos, Courtney Finch, and Luis Martínez-Sobrido

Abstract

Annual influenza epidemics are caused not only by influenza A viruses but also by influenza B viruses. Initially established for the generation of recombinant influenza A viruses, plasmid-based reverse genetics techniques have allowed researchers the generation of wild type and mutant viruses from full-length cDNA copies of the influenza viral genome. These reverse genetics approaches have allowed researchers to answer important questions on the biology of influenza viruses by genetically engineering infectious recombinant viruses. This has resulted in a better understanding of the molecular biology of influenza viruses, including both viral and host factors required for genome replication and transcription. With the ability to generate recombinant viruses containing specific mutations in the viral genome, these reverse genetics tools have also allowed the identification of viral and host factors involved in influenza pathogenesis, transmissibility, host-range interactions and restrictions, and virulence. Likewise, reverse genetics techniques have been used for the implementation of inactivated or live-attenuated influenza vaccines and the identification of anti-influenza drugs and their mechanism of antiviral activity. In 2002, these reverse genetics approaches allowed also the recovery of recombinant influenza B viruses entirely from plasmid DNA. In this chapter we describe the cloning of influenza B/Brisbane/60/2008 viral RNAs into the ambisense pDP-2002 plasmid and the experimental procedures for the successful generation of recombinant influenza B viruses.

Key words Influenza B virus, Plasmid-based reverse genetics, Virus rescue approaches, Recombinant influenza B virus, Ambisense plasmids

1 Introduction

Influenza A and B viruses belong to the *Orthomyxoviridae* family of segmented, single-stranded, negative-sense RNA viruses [1]. Influenza A and B viruses are important human pathogens that represent an important public health problem [2]. Influenza A virus has a broad species tropism and mainly exists in the wild aquatic fowl reservoir, whereas influenza B virus is primarily limited and adapted to the human population, although rare infections of seals have also been documented [3, 4]. While both influenza A and B viruses can cause influenza disease and seasonal epidemics, only influenza A viruses have been responsible for

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pandemics [5, 6]. Currently, only H1N1 and H3N2 subtypes of influenza A virus, and influenza B virus, are circulating in humans. To date, two major lineages of influenza B viruses can be found infecting humans, the Victoria-like and Yamagata-like strains that are divergent from the ancestral influenza B/Lee/1940 virus, which have been co-circulating in the human population since the 1980s [3, 33, 34]. Though influenza B viruses usually contribute less to epidemics than influenza A viruses of the H3N2 subtype, they contribute more than type A H1N1 influenza strains and are the predominant circulating strain once every 3 years [7–9]. Moreover, during the last decade, influenza B virus has been the cause of several acute respiratory illness outbreaks on cruise ships [10, 11], in schools [12–14], and within the military [15]; non-respiratory clinical outcomes [9, 16–23], and secondary bacterial pneumonia infections [22, 24, 25]. Influenza B virus health concerns are aggravated by their efficient transmission [26] and the lack of antiviral effectiveness [27]. Both trivalent inactivated vaccines (TIV) and live-attenuated influenza vaccines (LAIV) historically consist of three strains of influenza: two subtypes of influenza A (H1N1 and H3N2) plus one influenza B (Yamagata or Victoria lineages). However, a recent quadrivalent vaccine formulation containing representative strains of influenza B lineages has been implemented [28].

Influenza A and B viruses share many features, but they differ in their host range, virion structure, genomic organization, and glycan-binding specificities [23, 29]. The genomes of influenza A and B viruses consist of eight negative-sense, single-stranded RNA segments that share a common organization: a central coding region, sometimes encoding more than one polypeptide, flanked by relatively short untranslated regions (UTRs) [23, 29] (Fig. 1). Despite having similar genomes that encode homologous proteins, influenza A and B viruses can be distinguished by the different lengths of proteins and noncoding regions that serve as promoters for viral genome replication and gene transcription [3, 30, 31]. They can also be distinguished by the presence of accessory proteins encoded from overlapping open reading frames (ORFs) and by the antigenic differences of internal proteins [32]. For instance, influenza A and B viruses both encode ion channel proteins from the M gene (or segment 7), M2 and BM2, respectively. Uniquely, influenza A virus expresses the PB1–F2 pathogenicity factor, and influenza B virus expresses the NB ion channel, which is absent in type A influenza viruses [1]. However, both influenza A and B viruses encode two surface glycoproteins: hemagglutinin (HA), which is responsible for viral binding and fusion, and neuraminidase (NA), which is necessary for virus release from infected cells (Fig. 1).

The advent of reverse genetics and molecular engineering of recombinant viruses has transformed the virology field by permitting study of genetic changes in virus genomes. Because of

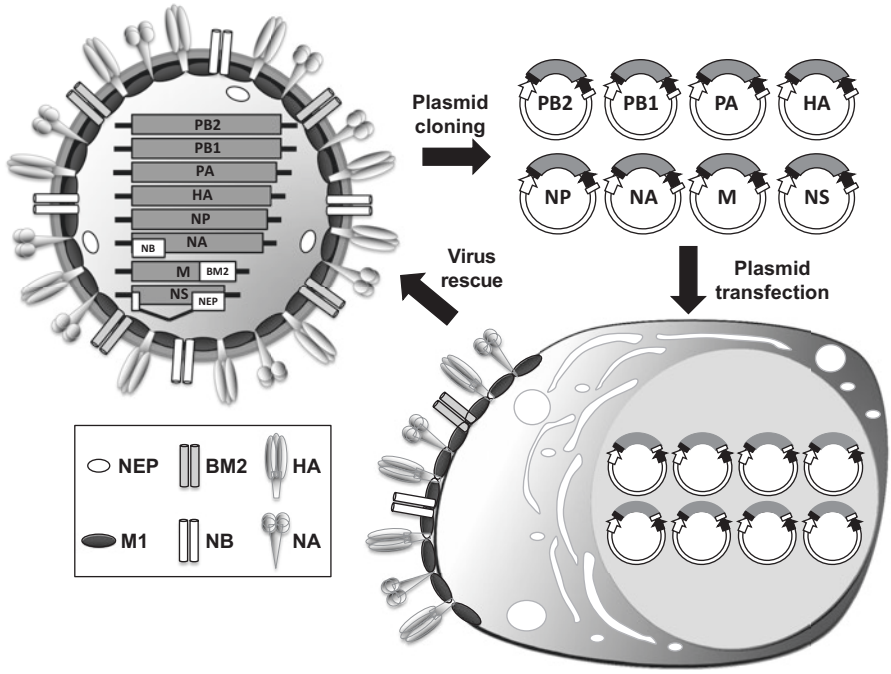

Fig. 1 Influenza B virion structure and plasmid-based reverse genetics: Influenza B virus is surrounded by a lipid bilayer containing the two viral glycoproteins Hemagglutinin (HA) and Neuraminidase (NA), and the ion channel proteins BM2 and NB. HA is the viral attachment protein responsible for binding to sialic acid-containing receptors. NA is responsible for viral release from infected cells. Underneath the lipid bilayer is a protein layer composed of the inner surface envelope matrix protein 1 (M1) which plays a role in virion assembly and budding, and the nuclear exporting protein (NEP) required for nuclear export of the eight viral ribonucleoprotein (vRNP) complexes. Influenza B virus contains eight single-stranded, negative-sense, viral (v)RNA segments (PB2, PB1, PA, HA, NP, NA, M, and NS) encapsidated by the viral nucleoprotein (NP). Associated with the eight vRNP complexes are the viral PB2, PB1, and PA polymerase subunits that, together with the viral NP, are involved in viral genome replication and gene transcription (Fig. 2b). For the development of reverse genetics techniques, influenza B vRNAs are cloned into bidirectional plasmids. Transfection of the eight ambisense plasmids into permissible cells leads to rescue of recombinant influenza B virus. The eight influenza B virus genes (*gray boxes*) are represented in 3′ to 5′ negative sense. Alternative or overlapping open reading frames (ORFs) are also indicated (*white boxes*). Lines indicate the noncoding regions (NCR) at the 3′ and 5′ ends of each viral segment. The eight ambisense plasmids contain the influenza B viral cDNAs (*gray boxes*), the human polymerase I (hPol-I) promoter (*black arrow*), the mouse Pol-I terminator (*black box*), a polymerase II-dependent promoter (*white arrow*), and a polyadenylation sequence (*white box*). See main text and Fig. 2a for more information

influenza A viruses' pandemic potential [35–37], reverse genetics approaches for the generation of recombinant viruses were first established in 1999 for influenza A viruses [38, 39]. Three years later, in 2002, two groups independently reported the successful recovery of influenza B virus entirely from plasmid DNA [40, 41]. The ability to modify the genome of viruses has revolutionized the influenza research field, allowing specific questions to be answered by genetically engineered, infectious, recombinant viruses [42]. Studies have revealed details of virus genome replication and gene

transcription, virulence and pathogenicity, function of viral proteins, virus-host interaction, packaging signals, and host range and transmissibility [31, 32, 42, 43]. Moreover, reverse genetics technologies have also been implemented for the development of influenza virus vaccines [44] and for the generation of recombinant influenza viruses expressing foreign peptides and/or proteins as viral vaccine vectors [45–47].

In this chapter we describe the experimental procedures for the cloning of influenza B/Brisbane/60/2008, which belongs to the B/Victoria lineage, into the ambisense pDP-2002 plasmid for the successful generation of recombinant wild type or mutant viruses upon transfection into susceptible cell lines (Fig. 1).

2 Materials

2.1 Influenza B Virus

This protocol is described for the rescue of influenza B/Brisbane/60/2008 virus that, like other influenza B virus reverse genetics, can be performed under BSL-2 laboratory conditions (*see* **Note 1**). All material used for the rescue of influenza B/Brisbane/60/2008 should be sterilized before disposal, following the appropriated institutional biosafety committee (IBC) recommendations.

2.2 Influenza B vRNA Extraction

Precautions for handling RNA samples include purchasing reagents that are free of RNases, separating reagents and materials (tips, tubes, ddH_2O, etc.) for RNA work only, and wearing clean gloves.

1. TRIzol reagent (Invitrogen) or RNeasy Mini Kit (Qiagen) (*see* **Note 2**).
2. Chloroform (J.T. Baker).
3. Isopropyl alcohol (J.T. Baker).
4. Diethylpyrocarbonate (DEPC).
5. Ethanol (Koptec): Prepare a 75% ethanol solution in RNAse-free (ThermoFisher) or DEPC-treated ddH_2O.
6. DEPC-treated ddH_2O: Add 1–2 mL of DEPC to 900 mL of ddH_2O. Shake and incubate overnight in a fume hood. Autoclave the solution to inactive the remaining DEPC.
7. Laboratory equipment: Microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, water bath or heat block and microcentrifuge tubes.

2.3 cDNA Synthesis

1. SuperScript II Reverse Transcriptase (Invitrogen).
2. RNase inhibitor (Promega).
3. PCR Nucleotide Mix (dNTP; Roche).
4. RNAse-free (ThermoFisher) or DEPC-treated ddH_2O.
5. Laboratory equipment: Thermocycler and PCR tubes.

2.4 Amplification of Influenza B vRNAs

1. Expand high fidelity polymerase (Roche).
2. PCR Nucleotide Mix (dNTP; Roche).
3. RNAse-free (ThermoFisher) or DEPC-treated ddH_2O.
4. SeaKem LE agarose (Lonza) (*see* **Note 3**).
5. Wizard SV Gel and PCR Clean-up system (Promega).
6. Laboratory equipment: Thermocycler, electrophoresis system, power supply, NanoDrop (or similar spectrophotometer) and PCR tubes.

2.5 Cloning Influenza B cDNAs

1. BsmBI restriction enzyme (New England Biolabs Inc.).
2. Shrimp Alkaline Phosphatase, SAP (Promega).
3. T4 DNA Ligase (Promega).
4. DH5α- or JM109-competent cells (Invitrogen).
5. Luria-Broth (LB) liquid media.
6. Super Optimal broth with Catabolite repression (SOC) media.
7. LB agar plates.
8. Ampicillin powder (Sigma).
9. Wizard SV Gel and PCR Clean-up system (Promega).
10. SeaKem LE agarose (Lonza) (*see* **Note 3**).
11. Laboratory equipment: Microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, a water bath or heat block, an electrophoresis system, a power supply, a NanoDrop (or similar spectrophotometer), petri dishes, a microbiological incubator and microcentrifuge tubes.

LB liquid media: LB media can be purchased from suppliers (ThermoFisher) or prepared in the laboratory. To prepare LB liquid media, mix 10 g of Bacto Tryptone (BD), 5 g Bacto Yeast extract (BD), and 10 g NaCl (Sigma) in 900 mL of ddH_2O. Adjust the pH to 7.0 with 5 N NaOH and adjust the volume of the solution to 1 L with ddH_2O. Autoclave and store at 4 °C. To prepare LB liquid media with Amp for growing DH5α-competent cells transformed with the influenza B/Brisbane/60/2008 pDP-2002 plasmids, add 1 mL of the ampicillin stock (1000×) to 1 L of LB liquid media (final ampicillin concentration 100 μg/mL). LB liquid media with ampicillin can be stored at 4 °C.

SOC liquid media: SOC media can be purchased (ThermoFisher) or prepared in the laboratory. To prepare SOC media, mix 20 g of Bacto Tryptone (BD), 5 g Bacto Yeast extract (BD), 0.5 g NaCl (Sigma), and 2.5 mL of 1 M KCl (final concentration 2.5 mM) in 900 mL of ddH_2O. Adjust the pH to 7.0 with 5 N NaOH and adjust the volume of the solution to 1 L with ddH_2O. Autoclave and before use, add 10 mL of 1 M $MgCl_2$ (final

concentration 10 mM) and 20 mL of 1 M glucose (final concentration 20 mM). Store SOC media at 4 °C

LB agar plates: LB plates can be purchased from suppliers (Sigma) or prepared in the laboratory. Prepare 500 mL LB liquid media and, just before autoclaving, add 15 g/L of Bacto Agar (Fisher Scientific). After autoclaving, equilibrate at 55 °C for 30 min and add 500 μL of ampicillin 1000×. Add 20–25 mL to Petri dishes. Store LB agar plates at 4 °C.

Ampicillin 1000×: We recommend preparing a stock solution of ampicillin at 100 mg/mL (1000×) and storing it in 500 μL aliquots at −20 °C.

2.6 Ambisense Plasmids for the Generation of Recombinant Influenza B Viruses

Plasmid pDP-2002 (Fig. 2a) is a derivative of pHW2000 [48]. This bidirectional plasmid contains two transcription units in opposite orientation. The first unit is the human polymerase I (hPol-I) promoter and a murine Pol-I transcription terminator (TI) for the expression of influenza B vRNAs. Expression from the hPol-I cassette generates vRNAs without additional nucleotides at the 3′ and 5′ end of the viral genome that are recognized by the influenza B viral NP and polymerase complex (PB2, PB1, and PA) for viral genome replication (cRNA) and gene transcription (mRNA) (Fig. 2b). The second unit is the polymerase II-driven cytomegalovirus promoter (pCMV) and the bovine growth hormone polyadenylation signal (aBGH) to express mRNAs (Fig. 2b). The use of this combined RNA Pol-I/II approach allows vRNA and mRNA syntheses from the same vector, eliminating the need for separate vRNA and mRNA plasmids and, therefore, reducing the number of plasmids to eight for the efficient rescue of influenza B viruses [32, 42] (*see* **Note 4**). Influenza B viral cDNAs are cloned into the pDP-2002 plasmid using two BsmBI restriction sites located between both transcription cassettes (Fig. 2a). The bidirectional pDP-2002 plasmid contains a spacer sequence of 444 nucleotides between the two BsmBI restriction sites that can be used to monitor BsmBI digestion efficiency using agarose gel electrophoresis.

Plasmids are prepared using commercially available plasmid preparation kits, following the manufacture's recommendations. We suggest storing the plasmids at −20 °C at a concentration of 1.0–0.5 mg/mL. This significantly simplifies the procedure to generate recombinant influenza B wild type or mutant viruses since 1–2 μL, respectively, of each plasmid is then used for viral rescue (*see* **Note 5**).

2.7 Mammalian Cell Lines for the Generation of Recombinant Influenza B Viruses

1. Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection, ATCC, CRL-11268) (*see* **Note 6**): A derivative of 293 cells containing the temperature-sensitive Simian virus 40 (SV40) T antigen [49]. The pDP-2002 plasmid drives expression of influenza B/Brisbane/60/2008

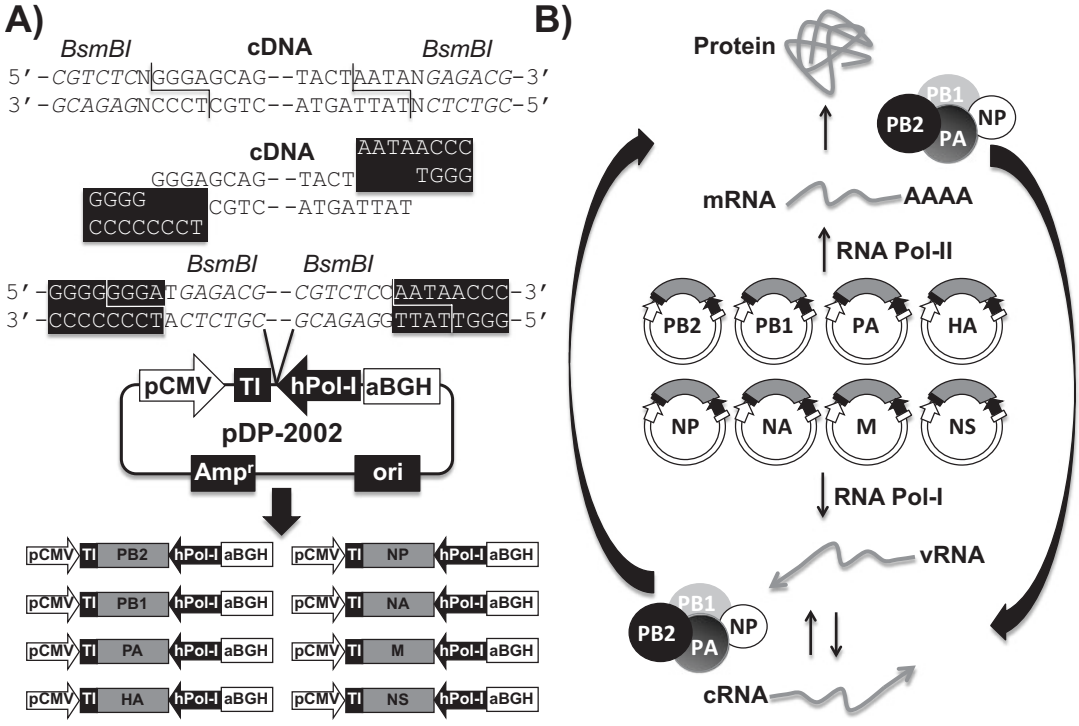

Fig. 2 Influenza B vRNA cloning into the ambisense pDP-2002 plasmid. (**a**) Schematic representation of the pDP-2002 plasmid, influenza B cDNA inserts and generation of influenza B rescue plasmids: The pDP-2002 plasmid, derived from pHW2000 [48], is a bidirectional vector containing the human polymerase I promoter (hPol-I, *black arrow*) and the mouse Pol-I terminator (TI, *black box*) sequences to direct the synthesis of the influenza B vRNAs. Transcription from the Pol-I cassette results in vRNAs identical to those present in influenza B virus, allowing their recognition by the influenza polymerase complex. In opposite orientation to the Pol-I cassette, a polymerase II-dependent cytomegalovirus promoter (pCMV, *white arrow*) and a polyadenylation sequence (aBGH, *white box*) direct the synthesis of influenza B proteins from the same viral cDNA. Influenza B vRNAs are amplified by RT-PCR with oligonucleotides containing BsmBI restriction sites (*italic*). BsmBI digested RT-PCR cDNA products are ligated into the pDP-2002 BsmBI-digested plasmid containing same nucleotide overhangs, to generate the eight influenza B ambisense plasmids for efficient virus rescue (*bottom*). Within this pDP-2002 plasmid, influenza B cDNAs are flanked by the Pol-I (for production of vRNAs) and Pol-II (for production of viral proteins) cassettes. Because of the nature of the BsmBI, restriction sites are eliminated after insertion of influenza B cDNAs. (**b)** Influenza B plasmid-based reverse genetics: In cells transfected with the influenza B ambisense pDP-2002 plasmids, the Pol-I cassette generates the eight negative sense vRNAs while the Pol-II direct the synthesis of the eight viral mRNAs that are translated into the influenza B viral proteins. After translation, influenza B NP and the polymerase complex PA, PB1 and PB2 associate with the vRNA to form the viral ribonucleoprotein (vRNP) complexes and initiates transcription from the viral promoter located within the noncoding regions at the 3′ termini of the vRNAs. Transcription results in the synthesis of more influenza B mRNAs and proteins. The influenza B polymerase complex also replicates the vRNAs into complementary (c) RNAs that serve as templates for the amplification of vRNAs. Newly synthesized vRNAs, together with the structural viral proteins results in the formation of new influenza B viruses. See main text for more information

RNAs based on the use of hPol-I promoter. Activity of Pol-I promoters has been shown to be species specific [50, 51] and, therefore, requires the use of a human cell line. HEK293T cells have been shown to have high transfection efficiency and thus, optimal for initial amplification of influenza B vRNAs from ambisense plasmids [40, 41]. Although highly transfectable, HEK293T cells are not ideal for influenza B virus replication.

2. Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) (*see* **Note 6**): This cell line is less optimal for transfection than HEK293T cells. Moreover, because of the species specificity of the Pol-I promoters [50, 51], transfection of MDCK cells with the eight influenza B pDP-2002 ambisense plasmids will not result in the initial amplification of vRNAs. However, MDCK cells are the best cell line for influenza B virus replication, producing high viral titers [52].

Both cell lines are maintained with DMEM 10% FBS, 1% PSG at 37 °C in a 5% CO_2 atmosphere tissue culture humidified incubator. For virus rescue and amplification, cells are maintained in DMEM 0.3% BSA, 1% PSG (or OptiMEM-I media) with 1 μg/mL of TPDCK-treated trypsin at 33 °C in the 5% CO_2 atmosphere tissue culture humidified incubator.

2.8 Tissue Culture Media and Reagents

1. Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Inc).
2. Fetal bovine serum (FBS; Atlanta Biologicals).
3. 35% Bovine Serum Albumin (BSA; Sigma).
4. Penicillin/Streptomycin (100 μg/mL)-2 mM L-Glutamine (PSG; Mediatech, Inc).
5. Trypsin–EDTA (Gibco).
6. Phosphate buffered saline (PBS).
7. Lipofectamine 2000 (LPF2000; Invitrogen).
8. OptiMEM-I medium (Invitrogen).
9. Tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma).
10. Tissue culture material (Greiner Bio-one).
11. Laboratory equipment: Centrifuge and microcentrifuge tubes, laboratory 5% CO_2 tissue culture humidified incubators at 33 °C (virus rescue and amplification) and 37 °C (maintenance of cell lines).

DMEM 10% FBS, 1% PSG: This media is used for maintenance of both HEK293T and MDCK cells. Mix 445 mL DMEM, 50 mL of heat inactivated FBS, and 5 mL of 100× PSG. Store at 4 °C.

DMEM 0.3% BSA, 1% PSG: This postinfection media is used after viral infections. Mix 490.7 mL of DMEM, 4.3 mL of 35%

BSA, and 5 mL of PSG. Store at 4 °C. Just before use, add TPCK-treated trypsin to a final concentration of 1 μg/mL (1:1000 dilution of the TPCK-treated trypsin stock at 1 mg/mL).

PBS 10× (Phosphate buffered saline): To prepare PBS 10×, mix 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4. Adjust pH to 7.4. Add ddH_2O up to 1 L and sterilize by autoclaving. PBS 10× can be stored at room temperature.

PBS 1×: dilute 1:10 PBS 10× with ddH_2O. Sterilize by autoclaving. Store at room temperature.

PBS 1×, 0.3% BSA, 1% PSG: This media is used for viral dilutions and during viral infections. Remove 13 mL from a 500 mL sterile PBS 1× bottle. Add 5 mL of 100× PSG and 3 mL of 35% BSA. Mix and add 5 mL of Ca/Mg 100× for a total of 500 mL. Make 100 mL aliquots and store at 4 °C.

Ca/Mg 100×: Mix 1.327 g of $CaCl_2 \cdot 2H_2O$ with 2.133 g of $MgCl_2 \cdot 6H_2O$. Add ddH_2O up to 100 mL. Sterilize by autoclaving. Store at room temperature.

2.9 Influenza B Virus Amplification/ Propagation in Eggs

1. Specific Pathogen-Free (SPF) eggs: Embryonated 8–10-day-old chicken eggs can be obtained from Charles River Laboratories or another supplier. Eggs can be incubated at 37 °C before virus rescue but at 33 °C for amplification, propagation, and growth of influenza B virus.

   All procedures to infect and harvest the allantoic fluid of chicken embryonated eggs are performed under sterile conditions. Always, dispose infected eggs in biosafety autoclavable bags and autoclave following IBC recommendations.

   Verify the viability of the embryos before and after viral infection by candling the eggs (*see* **Note 7**). Prior to harvesting the allantoic fluid, incubate the eggs for at least 2 h (preferably overnight) at 4 °C to kill the chicken embryo and coagulate the blood. Alternatively, incubate eggs at −20 °C for 30 min before harvesting. Approximately 8–10 mL of allantoic fluid can be harvested from 8–10-day-old chicken embryonated eggs.

   In some instances, it is difficult to obtain chicken embryonated eggs for rescue or amplification of influenza B virus. We have been successful in both, the rescue and the generation of virus stocks of influenza B virus using tissue culture monolayers of MDCK cells.
2. Ethanol 75%.
3. 1 and 5 mL syringes (BD).
4. Paraffin wax (Anachemia).
5. Laboratory equipment: Humidified egg incubator (*see* **Note 8**) and egg candler (Lyon electric company, Inc).

2.10 Methods for the Detection of Influenza B Virus

2.10.1 HA Assay

1. V-bottom 96-well plate (Nunc).
2. Turkey or chicken red blood cells (RBCs).
3. PBS 1×.
4. 50 mL centrifuge tubes (Greiner bio-one).

Preparation of 0.5–1.0% turkey or chicken RBCs: Turkey or chicken RBCs are purchased from a supplier (Biolink) and provided in Alselver's solution, which should be removed prior to their use. Mix 5 mL of turkey or chicken RBCs with 45 mL of PBS 1× in a 50 mL centrifuge tube. Pellet the RBCs by centrifugation at 200 × *g* for 5 min at room temperature. Carefully remove the supernatant. The remaining RBCs are considered to be at, approximately, 100%. Use a 1:100 or a 1:200 dilution of 100% RBC in PBS 1× for a final concentration of 1% or 0.5% RBCs, respectively (*see* **Note 9**).

2.10.2 Immunofluorescence (IF) Assay

1. 10% Formaldehyde (Polysciences).
2. Triton X-100 (Sigma).
3. BSA (Sigma).
4. PBS 1×.
5. Primary antibody against influenza B nucleoprotein, NP (Abcam; ab20711).
6. Anti-mouse IgG-FITC conjugate (Dako).
7. 96-well tissue culture plates (Greiner bio-one).
8. Laboratory equipment: Fluorescent microscope, centrifuge, multichannel pipette, and hemocytometer with coverslip.

Fix and permeabilization solution: Mix 400 mL of 10% formaldehyde, 5 mL of Triton X-100, and 595 mL of ddH_2O.

Blocking solution: To prepare the blocking solution, dissolve 25 g of BSA in 1 liter of PBS 1×.

Primary and secondary antibody dilution: Dilute the primary or secondary antibody at 1–2 μg/mL and 1:200, respectively, in blocking solution.

2.10.3 Plaque Assay

1. 10% formaldehyde (Polysciences).
2. 0.5% crystal violet (Fisher Scientific).
3. DMEM/F-12 powder (GIBCO).
4. 5% $NaHCO_3$.
5. HEPES solution 1 M (GIBCO).
6. 2% agar (Oxoid).
7. 1% DEAE-Dextran (Pharmacia).
8. PSG 100×.
9. 6-well tissue culture plates (Greiner bio-one).

10. Laboratory equipment: Light microscope, centrifuge, water bath, microwave oven, and hemocytometer with coverslip.

DMEM-F12 2×: This media is used for influenza B virus plaque assays. Add the content of 1 bag of DMEM-F12 powder in 440 mL of ddH_2O. Add 10 mL of PSG 100×, 6 mL of 35% BSA, 10 mL of HEPES solution 1 M, and 24 mL of 5% $NaHCO_3$. Sterilize by filtration and store at 4 °C.

DMEM-F12/Agar mixture: For a total volume of 50 mL, mix 8.5 mL of ddH_2O, 25 mL of DMEM-F12 2×, 0.5 mL of 1% DEAE-Dextran, 1 mL of 5% $NaHCO_3$, and 15 mL of 2% Agar.

2% Agar: Mix 2 g of agar in 100 mL of ddH_2O. Sterilize by autoclave. Store at room temperature. Melt in the microwave for 1–2 min before using.

1% DEAE-Dextran: Dissolve 1 g of DEAE-Dextran in 100 mL of ddH_2O. Sterilize by autoclaving and store at 4 °C.

5% NaHCO3: Dissolve 5 g of $NaHCO_3$ in 100 mL of ddH_2O. Sterilize by autoclaving and store at 4 °C.

2.5% formaldehyde: Dilute the 10% formaldehyde in PBS 1×.

0.5% Crystal violet solution: Dissolve 0.5 g of crystal violet in 20% methanol. Store at room temperature. Crystal violet is a powerful staining solution. Handle it carefully.

2.10.4 Tissue Culture Infectious Dose 50 ($TCID_{50}$) Assay

1. 10% formaldehyde (Polysciences).
2. 0.5% crystal violet (Fisher Scientific).
3. 96-well tissue culture plates (Greiner bio-one).
4. Laboratory equipment: Light microscope, centrifuge and hemocytometer with coverslip.

3 Methods

3.1 Extraction of Influenza B vRNA

Ribonucleases (RNAses) are enzymes that catalyze the degradation of RNA and are commonly found on work surfaces and skin [53]. Therefore it is recommended to wear gloves and work using sterile conditions during the extraction and manipulation of RNA. We also recommend keeping the sample(s) on ice throughout the entire RNA extraction procedure to avoid RNA degradation. In addition, it is recommended to always use sterile, disposable plasticware and use pipettes which are dedicated only for RNA handling.

1. vRNAs are extracted using TRIzol reagent (Invitrogen), RNeasy Mini Kit (Qiagen), or any other available RNA extraction method following procedures recommended by the manufacturer (*see* **Note 10**). High viral titer samples are typically required for efficient amplification of vRNAs. Clinical material is usually not reliable for amplification of the influenza B full-length genes. Thus, amplification of influenza B virus in tissue cultured

Table 1
Reverse Transcriptase (RT) step and PCR conditions for the amplification of influenza B/Brisbane/60/2008 vRNAs

Component	Volume
RT step	
Total RNA	1–10 μL (0.1–2.0 μg)
dNTPs (10 mM)	1 μL
Primer (10 **μ**M)	1 μL
ddH_2O	Up to 12 μL
5× Buffer	4 μL
0.1 M DTT	2 μL
RNaseOUT	1 μL
SuperScript II RT	1 μL
PCR step	
cDNA (from RT step)	2 μL
dNTPs (10 mM)	2 μL
Forward primer (10 **μ**M)	2.5 μL
Reverse primer (10 **μ**M)	2.5 μL
10× Buffer	5 μL
$MgCl_2$ (25 mM)	6 μL
High fidelity polymerase	0.75 μL
ddH_2O	29.25 μL
PCR cycles	
No. cycles	Temperature/time
1	94 °C/2 min
30	94 °C/30 s. 37–55 °C/30 s 72 °C/2–3 min
1	72 °C/10 min

MDCK cells or 8–10-day-old chicken embryonated eggs is recommended.

2. Extracted RNAs are eluted or resuspended in RNase-free ddH_2O. RNA concentration is quantified using a NanoDrop or similar spectrophotometer (*see* **Note 11**). RNA can be stored at −80 °C until cDNA synthesis.

3.2 cDNA Synthesis of Influenza B vRNAs

1. To convert vRNA into cDNA, we use SuperScript II reverse transcriptase, Invitrogen (*see* **Note 12**).
2. Mix the components total RNA, dNTPs, primer, and ddH2O on ice as indicated in Table 1. Primers used for the synthesis of influenza B/Brisbane/60/2008 cDNAs are indicated in Table 2. An individual RT-PCR reaction for each viral segment has to be done in order to convert the eight influenza B/Brisbane/60/2008 vRNAs into cDNAs (*see* **Note 13**).

Table 2
Oligonucleotides for the amplification of influenza B/Brisbane/60/2008 vRNAs: forward and reverse primers for the amplification of influenza B/Brisbane/60/2008 PB2, PB1, PA, HA, NP, NA, M, and NS vRNA segments are indicated

Segment	Forward primer	Reverse primer
PB2	AATT*CGTCTC*AGGGAGCAGAAGC GGAGCGTTTTCA	AATT*CGTCTC*GTATTAGTAGAAACA CGAGCATTTTT
PB1	AATT*CGTCTC*AGGGAGCAGAAGC GGAGCCTTTAAG	AATT*CGTCTC*GTATTAGTAGAAACA CGAGCCTTTTT
PA	AATT*CGTCTC*AGGGAGCAGAAGC GGTGCGTTTGAT	AATT*CGTCTC*GTATTAGTAGAAACA CGTGCATTTTT
HA	AATT*CGTCTC*AGGGAGCAGAAGC AGAGCATTTTCT	AATT*CGTCTC*GTATTAGTAGTAACA AGAGCATTTTT
NP	AATT*CGTCTC*AGGGAGCAGAAGC ACAGCATTTTCT	AATT*CGTCTC*GTATTAGTAGAAACA ACAGCATTTTTT
NA	AATT*CGTCTC*AGGGAGCAGAAGC AGAGCATCTTCT	AATT*CGTCTC*GTATTAGTAGTAACA AGAGCATTTTTC
M	AATT*CGTCTC*AGGGAGCAGAAGC ACGCACTTTCTT	AATT*CGTCTC*GTATTAGTAGAAACA ACGCACTTTTTC
NS	AATT*CGTCTC*AGGGAGCAGAAGC AGAGGATTTGTT	AATT*CGTCTC*GTATTAGTAGTAAC AAGAGGATTTTTA

Sequences complementary to influenza B/Brisbane/60/2008 vRNAs are shown in bold. Segment-specific sequences are underlined. BsmBI restriction sites in both forward and reverse primers are italicized

3. Incubate the mixture at 65 °C for 5 min and then at 4 °C for 2 min.
4. Add the remaining components shown in Table 1.
5. Incubate at 50 °C for 60 min, followed by 15 min at 70 °C.
6. The obtained cDNA can be used directly for PCR amplification of the viral segments or stored at −20 °C for further use.

3.3 PCR Amplification of Full-Length Influenza B Viral cDNAs

1. For the PCR amplification of influenza B cDNAs we use the Expand High Fidelity PCR system, Roche (*see* **Note 14**). Primers used in this protocol (Table 2) are for the amplification of the cDNAs from Influenza B/Brisbane/60/2008. However, the 3′ and 5′-terminal nucleotides of influenza B RNAs are highly conserved among the eight viral segments (*see* **Notes 13** and **15**). In the primers listed in Table 2, the highly conserved sequences are followed by segment-specific nucleotides that are also highly conserved among influenza B viruses. Forward and reverse primers contain sequences complementary to the viral segment together with a BsmBI restriction site (CGTCTCN 1/5, underlined) for cloning purposes (*see* **Notes 16** and **17**).

2. Prepare the PCR mix (50 μL reaction volume) as indicated in Table 1.
3. Add 2 μL of the cDNA reaction mix from Subheading 3.2.
4. Incubate the PCR cDNA mix as indicated in Table 1.
5. Analyze the PCR products by standard agarose gel electrophoresis. PCR reactions can be stored at 4 °C.
6. If a single PCR product of the expected size is detected, remove components of the Expand High Fidelity PCR system (polymerase, salts, dNTPs, and primers) using a commercial PCR cleanup kit (Promega). If multiple products are detected, separate the PCR products by gel electrophoresis, remove an agarose slice containing the required product, and use a gel extraction kit (Promega) to isolate the DNA from the agarose slice (*see* **Note 18**).
7. Elute the required PCR product from the PCR cleanup or the gel extraction kit columns in ddH_2O.
8. Quantify the nucleic acid concentration of the PCR product using a NanoDrop or similar spectrophotometer.
9. Store the PCR product at −20 °C until further use.

3.4 Cloning of Eight Influenza B Viral cDNAs into the Ambisense pDP-2002 Rescue Plasmid

To generate influenza B virus entirely from cloned cDNA, the eight individual viral genomic segments should be cloned into a bidirectional rescue plasmid [31, 40, 41]. Here we described the cloning of influenza B/Brisbane/60/2008 into the ambisense pDP-2002 (Fig. 2a) (*see* **Note 4**). In this plasmid, influenza B viral cDNAs are inserted between the human RNA polymerase I promoter (hPol-I) and the mouse terminator (TI) sequences (Fig. 2a) (*see* **Note 19**). This polymerase I transcription/terminator cassette is flanked by an RNA polymerase II-dependent (Pol-II) cytomegalovirus promoter (pCMV) and a polyadenylation site (aBGH) (Fig. 2a). The orientation of the two transcription units allows the synthesis of negative-sense vRNA from the hPol-I cassette, and positive-sense mRNA from the Pol-II unit, from one viral cDNA template (Fig. 2a).

Preparation of influenza B cDNA inserts and pDP-2002 vector:

1. Digest the PCR products (0.5–1 μg) from Subheading 3.3, **step 9**, and the vector pDP-2002 (2 μg) by adding 2 μL of BsmBI restriction enzyme and 5 μL of the BsmBI 10× buffer in a 50 μL reaction. Incubate the reaction mix at 55 °C for 2 h (*see* **Note 20**).
2. Identify the digested products by agarose gel electrophoresis. Cut the gel slice containing the digested products and purify using a commercial gel agarose cleanup kit (Promega). Elute the DNA in ddH_2O and quantify the DNA concentrations using a NanoDrop or similar spectrophotometer (*see* **Note 21**).

3. Dephosphorylate the BsmBI restriction-digested pDP-2002 vector to prevent self-ligation by treating with shrimp alkaline phosphatase (SAP) (1 U/μg DNA) at 37 °C for 60 min (*see* **Note 22**). Next, add 1 μL more of SAP and incubate for 20 min. Inactivate SAP by heating at 65 °C for 15 min. The dephosphorylated pDP-2002 vector can be purified using a commercial PCR cleanup kit (Promega). Quantify the DNA concentration using a NanoDrop or similar spectrophotometer. Insert and vectors may be used immediately for ligation or stored at −20 °C for later use.
4. Ligate the digested PCR products into the pDP-2002 vector by adding the following: 100 ng of digested and SAP-treated pDP-2002 vector, 300 ng of DNA insert (1:3 molar ratio is recommended), 2 μL 10× ligation buffer, 1 μL (5 U) T4 DNA ligase (Roche), and ddH_2O up to 20 μL (*see* **Note 23**). Incubate the ligation mixture at room temperature for 1 h.
5. Transform JM109- or DH5α-competent *E. coli* cells (*see* **Note 24**) by adding 10 μL of the ligation mixture to 100 μL competent cells on ice. Incubate for 5 min on ice. Then, heat-shock for 45–60 s at 42 °C in a water bath, and transfer them back to ice for 5 min. Add 400 μL of SOC medium (*see* **Note 25**). Incubate for 1 h in a 37 °C shaking (200 rpm) incubator. Plate 100 μL of bacteria in prewarmed LB agar plates with 100 μg/mL of ampicillin (*see* **Note 26**). Incubate the plates, inverted, overnight at 37 °C (*see* **Note 27**).
6. Next day, transfer individual bacteria colonies from the LB agar plates to tubes containing 4 mL of LB liquid media with 100 μg/mL of ampicillin (*see* **Note 28**). Incubate for 16–18 h in a 37 °C shaking (200 rpm) incubator (*see* **Note 27**).
7. After overnight incubation, purify the plasmid DNA using a miniprep kit (Qiagen), following the manufacture's recommendations (*see* **Note 29**). Analyze the purified plasmid DNAs by restriction analysis and agarose gel electrophoresis (*see* **Notes 30** and **31**). Alternatively, selected clones can be initially screened by colony PCR to determine the presence or absence of insert DNA into pDP-2002 before sequencing. If the digested pDP-2002 plasmids contain the expected influenza B viral segments, confirm their nucleotide composition by sequencing (*see* **Note 32**).
8. Once the pDP-2002 plasmids have been confirmed by sequencing and shown not to contain mutations, make high-quality plasmid DNA preparations for mammalian transfection. Inoculate positive clones in 2 mL of LB liquid media containing 100 μg/mL of ampicillin and grow them at 37 °C for 8 h (*see* **Note 27**). Transfer the 2 mL bacteria solution to 250–500 mL of LB liquid media containing 100 μg/mL of ampicillin and grow at 37 °C for 16–18 h (*see* **Note 27**). Extract plasmid

DNA using a maxiprep DNA kit (Qiagen), according to the manufacturer's recommendations (*see* **Notes 29**, **33,** and **34**). Determine plasmid DNA concentration using a NanoDrop or similar spectrophotometer. We recommend evaluating the quality and quantity of the purified plasmid also by restriction digestion and agarose gel electrophoresis (*see* **Notes 5**, **30,** and **31**).

3.5 Transfection of Mammalian Cells for the Generation of Recombinant Influenza B Virus from Cloned cDNAs

Overall, rescue of recombinant influenza B viruses using plasmid DNA is a simple process. However, there are some key factors for a successful recovery of influenza B viruses using plasmid-based reverse genetics techniques: (1) good preparations of pDP-2002 plasmids, (2) proper maintenance of the HEK293T and MDCK cell lines, and (3) high DNA plasmid transfection efficiencies.

To increase the likelihood of successful influenza B virus rescue, we recommend three independent transfections. If more than one recombinant influenza B virus rescue is attempted, scale the following steps accordantly to the number of viruses to be rescued. The following transfection and infection protocol is established for 6-well plates. A schematic representation of the protocol to generate recombinant influenza B viruses is illustrated in Fig. 3.

1. *OptiMEM I-Lipofectamine 2000 (LPF2000) mix:* Prepare 250 μL of OptiMEM I media and 4–8 μL of LPF2000 (1 mg/mL) per transfection and incubate for 5–10 min at room temperature (*see* **Note 35**). Meanwhile, prepare the plasmid transfection mixture.
2. *Plasmid transfection mixture:* Add 1–2 μL (1.0–0.5 μg/μL) of each influenza B/Brisbane/60/2008 pDP-2002 plasmid (PB2, PB1, PA, HA, NP, NA, M, and NS) to a tube containing 50 μL of OptiMEM I media (*see* **Note 36**).
3. *OptiMEM I-LPF2000-plasmid DNA transfection mixture:* Add 250 μL of the OptiMEM I-LPF200 mix (**step 1**) into the plasmid DNA transfection mixture (**step 2**). Incubate for 20–30 min at room temperature. Meanwhile, prepare the human HEK293T and the canine MDCK cells for transfection.
4. *Preparation of HEK293T/MDCK co-culture:* Before starting, prewarm the PBS 1×, cell culture media (DMEM, 10% FBS, 1% PSG), and EDTA-trypsin mixture at 37 °C. Due to the species specificity of the RNA Pol-I promoter [50, 51], only cells from primate origin with high transfection efficiency, like HEK293T cells, can be used for virus rescue (*see* **Notes 37** and **38**). On the other hand, MDCK cells support the growth of many influenza viruses [52], including influenza B, and therefore are co-cultured with HEK293T cells for efficient viral rescues. Usually, one confluent 100 mm dish of each cell line (HEK293T and MDCK cells) can be used for approximately 10–12 viral rescues.

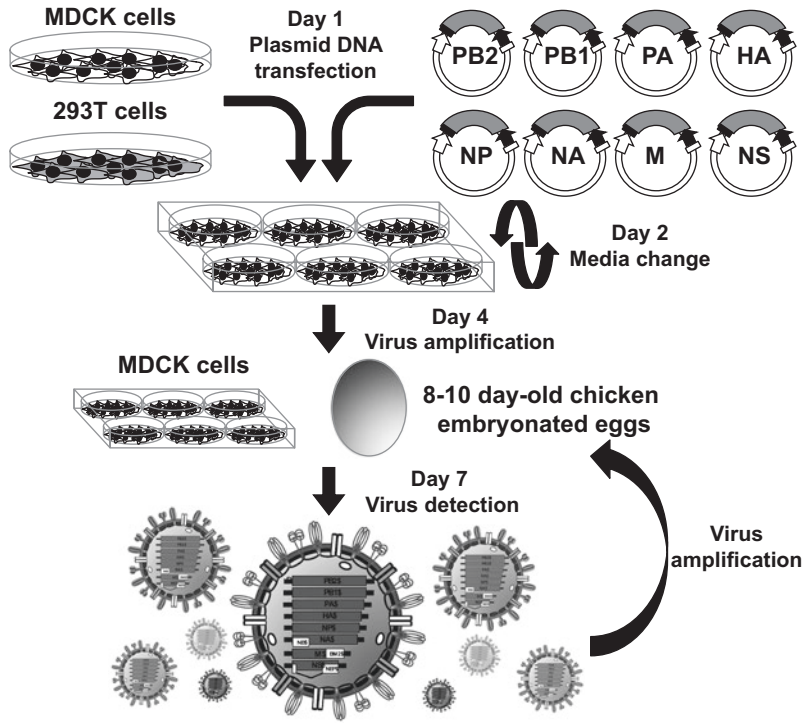

Fig. 3 Generation of recombinant influenza B virus using plasmid-based reverse genetics: The eight influenza B pDP-2002 ambisense plasmids (1 μg/plasmid) are transiently co-transfected in suspension, using LPF2000, into co-cultures of canine MDCK and human HEK293T cells (day 1, plasmid NDA transfection). Twenty-four hours after, transfection media is replaced by infection media (*see* **Note 43**) containing TPCK-treated trypsin (day 2, media change). Two days after, tissue culture supernatants are collected and used to infect fresh monolayers of MDCK cells or 8–10-day-old chicken embryonated eggs (day 4, virus amplification). Three days after virus amplification, tissue culture supernatants from MDCK cells or allantoic fluid from eggs are collected and examined for presence of influenza B virus using a hemagglutination (HA) assay (day 7, virus detection). If influenza B virus is not detected or detected at low titers, virus can be amplified by re-passaging the tissue culture supernatants or allantoic fluid from eggs into fresh MDCK cells and/or embryonated eggs (virus amplification). See text for detailed information

(a) Wash the cell monolayers twice with 4 mL of PBS 1×. Remove the last PBS 1× wash and add, slowly, 1 mL of a 0.25% Trypsin-EDTA. Incubate at 37 °C for 5–10 min until cells detach from the 100 mm dish plates (*see* **Note 39**).

(b) Carefully resuspend individually each cell line in 10 mL of cell culture media and transfer to a 15 mL centrifuge tube. Centrifuge both cell lines for 5 min at 200 × *g*.

(c) Carefully remove the cell culture media and resuspend the HEK293T and MDCK cells, individually, in 6 mL of cell culture media by gentle pipetting up and down (*see* **Note 40**). Determine the concentration of the MDCK cells using a hemocytometer. Mix equal volumes of HEK293T and

MDCK cells, based on the number of transfections, in a separate tube (*see* **Note 41**). This will give you the mixture of HEK293T and MDCK cells to be used for transfection (*see* **Note 42**).

5. After 20–30 min incubation at room temperature, add to each of the OptiMEM I-LPF2000-plasmid DNA transfection tubes (**step 3**) 1 mL of the HEK293T/MDCK cell co-culture and incubate for 5–10 min at room temperature.
6. Add the 1.3 mL (**step 6**) into individual wells in a 6-well tissue culture plate.
7. Gently shake the 6-well plate and let the transfection incubate for 6 h or overnight in the 33 °C, 5% CO_2 humidified tissue culture incubator.
8. After 6 h incubation (or the next day), change the transfection media for postinfection media containing 0.5 μg/mL of TPCK-treated trypsin and incubate for 48 h at 33 °C (Fig. 3) (*see* **Notes 43** and **44**).
9. After 48 h incubation, collect the tissue culture supernatants into microcentrifuge tubes and centrifuge for 1–2 min at 13,000 × *g* to remove cells and cell debris. Transfer supernatants to new microcentrifuge tubes. Tissue culture supernatants can be used immediately to infect fresh MDCK cells or 8–10-day-old chicken embryonated eggs or stored at −80 °C for later use.
10. Infection of MDCK cells or 8–10-day-old chicken embryonated eggs.

10.1 Infection of fresh MDCK cells:

(a) The day before infection, wash MDCK cells in 100 mm dishes twice with 4 mL of PBS 1×. Trypsinize the cells with 1 mL of 0.25% Trypsin-EDTA. Incubate at 37 °C for 5–10 min until cells detach. Resuspend the cells in 10 mL of cell culture media by gentle pipetting up and down and transfer to a 15 mL centrifuge tube. Centrifuge for 5 min at 200 × *g*. Remove supernatant and resuspend the cell pellet in 10 mL of cell culture media. Determine the concentration of the MDCK cells using a hemocytometer. Plate approximately 0.5×10^6 cells/well to reach confluence (1.0×10^6 cell/well) by the next day. Incubate at 37 °C in a 5% CO_2 humidified incubator (*see* **Note 45**).

(b) Before infection, check the MDCK cells under the microscope to confirm the presence of a cell monolayer (*see* **Note 46**).

(c) Aspirate the cell culture media and wash cells, twice, with 4 mL of PBS 1×.

(d) Infect the MDCK cells with 250 μL of virus-containing tissue culture supernatants (from **step 9**) for 1 h at room temperature. Gently rock the plates by hand every 10 min to prevent the cells from drying.

(e) After 1 h of viral absorption, remove the infection media and add 2 mL of postinfection media containing 1 μg/mL of TPCK-treated trypsin (*see* **Note 43**). Incubate the plates at 33 °C in a 5% CO_2 humidified incubator.

(f) At 3–4 days after passage, depending on the transfection efficiency and the virus load, cytopathic effect (CPE) will be observed in the MDCK-infected cells, which is indicative of virus rescue. However other assay(s) should be performed to confirm the presence of rescued virus (*see* Subheading 3.6). Harvest the tissue culture supernatants from MDCK cells when approximately 75% of the cells are infected as determined by CPE. Transfer the tissue culture supernatants to Eppendorf tubes and centrifuge at 13,000 × *g* for 5 min at room temperature to remove cells and cell debris. Collect the supernatants to fresh Eppendorf tubes and store them at 4 °C (short-term storage) or at −80 °C (long-term storage) (*see* **Note 47**).

10.2 Infection of 8–10-day-old chicken embryonated eggs:

All procedures to infect chicken embryonated eggs are performed under sterile conditions [42]. Before and after infection, it is very important to look for dead eggs, by candled them, before and after infection [42]. Before infection, a dead egg can be easily spotted by the absence of blood vessels as well as the absence of embryo mobility [42]. After viral infection, a dead egg will be easily identified by a bloody and smaller volume of allantoic fluid [42].

(a) Candle the 8–10-day-old chicken embryonated eggs using an egg candler and mark the inoculation site at the interphase between the allantoic cavity and the air sac. Spray the egg surface with 75% ethanol to maintain sterile conditions.

(b) With a 5 mL syringe needle, make a hole in the eggshell in the marked allantoic cavity/air sac interphase.

(c) With a 1 mL syringe, infect each egg with 100 μL of the virus-containing tissue culture supernatant (**step 9**).

(d) Cover the hole in the eggshell with melted wax applied with a sterile swab and incubate the eggs at 33 °C in a humidified incubator for 2–3 days.

(e) To harvest the allantoic fluid from chicken embryonated eggs, first place the eggs at 4 °C for at least 2 h (or overnight) to kill the chicken embryo and coagulate the blood. Alternatively, incubate eggs at −20 °C for 30 min before harvesting.

(f) Wash the eggshells with 75% ethanol to establish sterile conditions and open the egg, carefully, over the air cavity by tapping with a spoon.

(g) Remove the broken eggshell with the help of forceps.

(h) Using a 1 mL needle, remove the allantoic membrane without breaking the egg's yolk.

(i) Collect as much allantoic fluid as possible from each egg, without breaking or collecting any of the egg's yolk, with a sterile 10 mL plastic pipette. Transfer the allantoic fluid into a 15 mL centrifuge tube on ice (*see* **Note 48**). Use individual 15 mL centrifuge tubes for each egg.

(j) Centrifuge (200 × *g*) for 5 min at 4 °C to pellet any egg-derived red blood cells and transfer the clear allantoic fluid to a fresh 15 mL centrifuge tubes on ice.

(k) Allantoic fluid can be stored at 4 °C for a short period of time until evaluating the presence of rescued virus (*see* Subheading 3.6). Once the presence of virus in tissue culture supernatants or in the allantoic fluid of chicken embryonated eggs has been assessed, make aliquots the virus and place them at −80 °C for long-term storage (*see* **Note 47**). Virus titer can be determined as described in Subheading 3.6.

3.6 Methods for the Detection of Influenza B Rescued Virus

There are multiple direct and indirect methods for detecting and quantifying the presence of influenza B virus in tissue culture supernatants from MDCK cells or allantoic fluid from chicken embryonated eggs. All methods have unique advantages and disadvantages (not discussed here), and we focus on four typically used techniques (Fig. 4). Once an assay identifies the presence of rescued virus, the virus should be plaque purified and the genetic composition of the virus will need to be confirmed by RT-PCR and sequencing.

3.6.1 Hemagglutination (HA) Assay

This method is routinely used to detect the presence of rescued influenza B viruses in tissue culture supernatants from MDCK cells and/or the allantoic fluid from chicken embryonated eggs. We recommend performing, first, a hemagglutination (HA) assay since it takes approximately 30–60 min and it will provide information on the presence or absence of virus. In the case of influenza B virus, approximately 10^4 plaque forming units (PFUs) are required to give a positive signal in the HA assay. Therefore, other assays more sensitive than the HA assay (e.g., plaque, immunofluorescence, or $TCID_{50}$ assays) should be performed to confirm a true negative result. Presence of virus induces hemagglutination of turkey and/or chicken red blood cells (RBCs), while the absence of virus allows the formation of a red pellet in the bottom of the well (Fig. 4a). If virus is not detected using an HA assay because of low viral

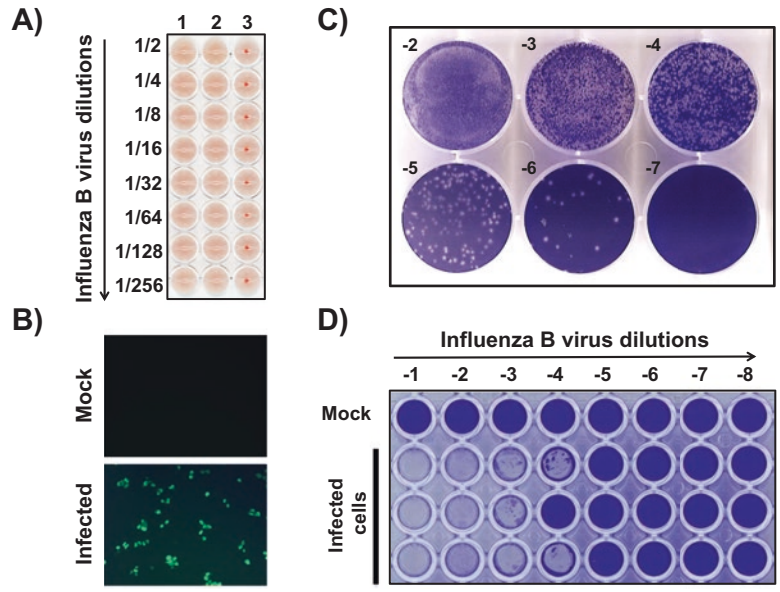

Fig. 4 Detection of influenza B virus. (**a**) Hemagglutination (HA) assay: Presence of influenza B virus from tissue culture supernatants or allantoic fluid from chicken embryonated eggs (Fig. 3) is evaluated by hemagglutination of turkey or chicken red blood cells (RBCs) macroscopically: absence of influenza B virus results in the formation of a red pellet (line 3) while presence of virus results in hemagglutination of RBCs (lines 1 and 2). (**b)** Immunofluorescence (IF) assay: IF assays are widely used for the rapid detection and quantification of influenza B virus infections in tissue culture supernatants or allantoic fluid from chicken embryonated eggs (Fig. 3), by determining the presence of virus antigens using specific antibodies. Confluent monolayers of MDCK cells (96-well plates, 4×10^5 cells/well) mock infected (*top panel*) and infected for 8–10 h with a series of influenza B virus dilutions (*bottom panel*, only a dilution example) are fixed, permeabilized, and stained with influenza B virus-specific antibodies and secondary fluorescein-conjugated antibodies. Presence of influenza B virus is determined by the presence of fluorescent positive cells. Viral titers using IFA are indicated as focus forming units (FFU)/ml. (**c**) Plaque assay: Plaque assays can be used to demonstrate the presence of rescued influenza B virus and/or to accurately determine the number of virus (plaque forming units, PFUs) from MDCK tissue culture supernatants of from the allantoic fluid of chicken embryonated eggs (Fig. 3). Confluent monolayers of MDCK cells (6-well plate format, 10^6 cells/well) are infected with tenfold serial dilutions (starting dilution of −2) of tissue culture supernatants or allantoic fluids. After 1 h viral absorption at room temperature, cell monolayers are overlaid with agar-containing medium. Influenza B virus plaques are visible at 48–72 h postinfection after staining with crystal violet. (**d**) Tissue culture infectious dose 50 ($TCID_{50}$) assay: $TCID_{50}$ is the tissue culture dilution dose required to infect 50% of monolayers of MDCK cells (96-well plate format, 4×10^5 cells/well). As shown in the figure, MDCK cells mock infected (top row) or infected (triplicates) with tenfold serial dilutions (starting dilution of −1) of tissue culture supernatants or allantoic fluids are evaluated 3–4 days postinfection for the presence of CPE using an inverted microscope and by staining with crystal violet. Influenza B viral titers are calculated based on the method of Reed and Muench [56]. For more details, see main text

titers, a new passage of virus-containing samples in fresh MDCK cells or 8–10-day-old embryonated chicken eggs will allow amplification of the rescued virus (Fig. 3). Tissue culture supernatants or allantoic fluids from this second passage should now be positive in the HA assay. It is important to include virus-containing tissue culture supernatants or allantoic fluids as a positive control of the HA assay. Likewise, mock-infected tissue culture supernatants, allantoic fluids, or PBS 1× should be included as negative control to validate the HA assay.

(a) Dispense 50 μL of PBS 1× into each well of a V-bottom 96-well plate.

(b) Add 50 μL of the tissue culture supernatants from MDKC cells or allantoic fluid from chicken embryonated eggs to the first well of the V-bottom 96-well plate, and make twofold serial dilutions (50 μL) for the following wells. Discard the extra 50 μL from the last well.

(c) Add 50 μL of 0.5–1.0% turkey or chicken RBCs (prepared in PBS 1×) to each well of the V-bottom 96-well plate (*see* **Note 49**).

(d) Incubate the V-bottom 96-well plate for 30–45 min on ice, until a red dot is visible in the bottom of the negative control sample.

(e) Read and interpret the results as shown in Fig. 4a.

3.6.2 Immunofluorescence (IF) Assay

This method is recommended, after performing an HA assay, to validate the identification of influenza B virus from tissue culture supernatants or allantoic fluid of chicken embryonated eggs due to the time (approximately 8–10 h) and sensitivity of the assay. IF assays can detect the presence of individual influenza B viruses in samples, thus it is possible that tissue culture supernatants from MDCK cells or allantoic fluids from chicken embryonated eggs could be negative in the HA assay (less than 10^4 PFU) yet positive in the IF assay. Direct IF assays use fluorescent-tagged anti-influenza B monoclonal or polyclonal antibodies (not discussed here) while indirect IF approaches require the use of unlabeled anti-influenza B monoclonal or polyclonal antibodies and fluorescently labeled secondary antibodies. Like for the HA assay, include negative and positive controls to validate the IF assay (*see* **Note 50**). All cell-based assays for the detection of influenza B virus (e.g., IFA, plaque, and $TCID_{50}$ assays) are recommended to be performed in MDCK cells.

(a) Seed approximately 2.0×10^4 MDCK cells/well, using a multichannel pipette, in 96-well plates the day before the infection to reach confluence (4×10^4 cell/well) after 1 day.

(b) Before infection, check the cells under a light microscope to confirm the presence of a cell monolayer.

(c) Make tenfold serial dilutions of the virus sample in PBS 1% BSA using a multichannel pipette. Starting with the most diluted sample, add 50 µL of the virus dilution to each of the wells in the 96-well plate with the multichannel pipette (*see* **Note 51**).

(d) Infect for 1 h at room temperature.

(e) After 1 h of viral absorption, remove the virus inoculum and add 100 µL of postinfection media containing 1 µg/mL of TPCK-treated trypsin with the multichannel pipette.

(f) Place the plates at 33 °C in the 5% CO_2 humidified incubator for 8–10 h (*see* **Note 52**).

(g) Remove the infectious media from the 96-well plates and add 100 µL/well of the fix/permeabilization solution with the multichannel pipette. Incubate at room temperature for 20 min.

(h) Remove the fix/permeabilization solution and wash once with 100 µL/well of PBS 1×, using the multichannel pipette.

(i) Incubate the cells with blocking solution for 1 h at room temperature (*see* **Note 53**).

(j) Dilute the primary unlabeled anti-influenza B monoclonal or polyclonal antibody in blocking solution (*see* **Note 54**). Add sufficient antibody to completely cover the cell monolayer to prevent drying (~100 µL/well). Incubate for 1 h at 37 °C.

(k) Carefully remove the primary antibody and wash the cells with 100 µL/well of PBS 1× three times using the multichannel pipette.

(l) Dilute the fluorescently labeled secondary antibody in blocking solution to a predetermined optimum working dilution. Add sufficient secondary antibody to completely cover the cell monolayer to prevent from drying (~100 µL/well). Incubate for 30 min at 37 °C.

(m) Carefully remove the secondary antibody and wash the cells with PBS 1× three times with the multichannel pipette (*see* **Note 55**). Remove the last PBS 1× wash and add 100 µL of PBS 1×.

(n) Evaluate the number of infected cells using a fluorescent microscope (Fig. 4d) and calculate the viral titer in focus forming units per milliliter (FFU/mL) using the following formula: FFU/mL = number of positive fluorescent cells × virus dilution × 1/volume of inoculum (mL).

3.6.3 Plaque Assay

This procedure is a direct quantitative measurement of the number of infectious viruses in tissue culture supernatants from MDCK cells or allantoic fluid from chicken embryonated eggs [54]. A semisolid overlay is placed over the MDCK cell monolayer to prevent the virus from infecting cells others than neighboring cells. Each arising plaque will have originated from a single virus. After 2–3 days postinfection, the cell monolayer is stained with 0.5% crystal violet to

visualize the plaques for quantification [44]. Presence of virus will lyse the cells and will result in the formation of a white plaque surrounded by a stained cell monolayer.

(a) The day before infection, seed approximately 5×10^5 cells/well in 6-well plates, to reach confluence (1×10^6 cells/well) the next day.

(b) Before infection, check the cells under a light microscope to confirm the presence of a cell monolayer.

(c) Make tenfold serial dilutions of the virus sample in PBS 1% BSA. Starting with the most diluted sample, add 500 μL of the virus dilution to each of the wells. Be careful not to track the pipette tip over the well, which could result in destroying the cell monolayer.

(d) Infect the cells for 1 h at room temperature, gently rocking the plates back and forth every 10 min by hand to prevent the cells from drying.

(e) During this incubation time, prepare the DMEM-F12 containing agar. Microwave the solution of agar for approximately 1–2 min and equilibrate at 42 °C in a water bath for 10 min. Mix the rest of the components of the DMEM-F12 media containing 1 μg/mL of TPCK-treated trypsin and warm them at 37 °C in a water bath. Mix the DMEM-F12 media with the equilibrated agar and gently add 2 mL to each of the wells in the 6-well plate. Let the plate(s) sit for 15 min at room temperature, as the agar overlay solidifies.

(f) Incubate the 6-well plate(s) at 33 °C in the 5% CO_2 humidified incubator in an inverted position to avoid condensation on the overlay agar.

(g) When viral plaques are visible (approximately 2–3 days), fix the plates with a solution of 2.5% formaldehyde in PBS 1× for 30 min at room temperature (or overnight). Carefully remove the overlay media and subsequently add 1 mL of 0.5% crystal violet solution to each well. Incubate for 30 min at room temperature.

(h) Remove the crystal violet solution and wash the cells with ddH_2O. Allow the plates to air-dry before counting the number of virus plaques.

(i) Count the plaques in the lowest two dilutions with visual plaques and calculate the viral titer in PFU/mL using the following formula: PFU/mL = number of plaques × virus dilution × 1/volume of inoculum (mL). An example of a plaque assay result is shown in Fig. 4c.

3.6.4 Tissue Culture Infectious Dose 50 ($TCID_{50}$) Assay

This procedure can be performed to determine the presence of virus and/or to titer the amount of virus from tissue culture supernatants or allantoic fluid [55]. It is based on the ability of

influenza B virus to cause CPE in monolayers of MDCK cells (Fig. 4d) [41]. We recommend performing the $TCID_{50}$ assay in triplicate (*see* **Note 51**). Similar to the previous assays, we recommend including positive and negative controls to validate the $TCID_{50}$ test.

(a) The day prior to infection, seed approximately 2.0×10^4 MDCK cells/well in 96-well plates using a multichannel pipette in DMEM 10% FBS, 1% PSG to reach confluence the day of infection.

(b) Before infection, check the cells under a light microscope to confirm the presence of a cell monolayer.

(c) On the day of infection, make tenfold serial dilutions of the virus sample (tissue culture supernatants or allantoic fluid) in infection media using a multichannel pipette.

(d) Starting with the most dilute sample, add 50 µL of the virus dilutions to each of the triplicate wells using the multichannel pipette. Be careful not to track the pipette tip over the well, which could result in destroying the cell monolayer.

(e) Infect for 1 h at room temperature.

(f) After 1 h of viral absorption, remove the virus inoculum and add 100 µL of postinfection media containing 1 µg/mL of TPCK-treated trypsin.

(g) Place the 96-well tissue culture plates at 33 °C in the 5% CO_2 humidified incubator.

(h) At 3–4 days postinfection, monitor CPE under a light microscope. Remove media and stain with 100 µL of the 0.5% crystal violet solution for 20 min at room temperature.

(i) Remove the crystal violet solution, wash the plates with ddH_2O, and let them dry at room temperature.

(j) Calculate the $TCID_{50}$ using the method of Reed and Muench [56]. An example of a plaque assay result is shown in Fig. 4d.

3.7 Amplification of Influenza B Virus and Production of Virus Stocks

As indicated above, it is possible that tissue culture supernatants from MDCK cells and/or allantoic fluid from chicken embryonated eggs contain low viral titers due to suboptimal transfection efficiencies, plasmid preparations or maintenance of the HEK293T and/or MDCK cells. In this case, influenza B virus should be amplified by infecting fresh monolayers of MDCK cells or the allantoic fluid of 8–10-day-old chicken embryonated cells (Fig. 3). Likewise, MDCK cells or 8–10-day-old chicken embryonated eggs should be used to generate influenza B virus stocks. Amplification of influenza B virus in MDCK cells or the allantoic fluid of chicken embryonated eggs follows the same protocol as described in Subheading 3.5, **step 10**. We recommend infecting MDCK cells with a multiplicity of infection (MOI) of 0.001 or chicken embryonated eggs with 10^2–10^3

PFUs and incubate them at 33 °C for 2–3 days. We also recommend storing the collected virus in small volume aliquots (0.1–0.2 mL) for long-term storage at −80 °C (*see* **Note 47**).

3.8 Identification of Recombinant Influenza B Virus Generated by Reverse Genetics

As described before, we usually include a genomic tag in one of the influenza B viral segments for easy identification of the recombinant virus. Recombinant viruses should be plaque purified and after amplification, RT-PCR and sequencing approaches should be performed to verify the recombinant nature of the rescued virus. Likewise, recombinant influenza B viruses containing mutations should be verified by RT-PCR and sequencing analyses (*see* **Note 56**).

4 Notes

1. Although in this chapter we describe plasmid-based reverse genetics for the generation of recombinant influenza B/Brisbane/60/2008 viruses [31], this protocol can be used for the recovery of other recombinant influenza B viruses.
2. Other available RNA extraction reagents and methods can also be used, following the manufacturer's recommendations.
3. Other agaroses can also be used for DNA gel electrophoresis.
4. Alternatively, other ambisense plasmids, such as pDZ [57] or pHW2000 [48], can be used for efficient influenza B virus rescues.
5. Plasmid preparation is one of the key factors for efficient influenza B virus rescues. We recommend having clean (a 260/280 ratio of >1.8 as determined by Nanodrop or similar spectrophotometer) plasmid preparations for successful attempts. Plasmid stocks at 1.0–0.5 mg/mL facilitate the generation of recombinant influenza B viruses since same volume (e.g., 1–2 μL, respectively) of the different ambisense plasmids is used for transfection of mammalian cells.
6. HEK293T and MDCK cells are also available from the Biodefense and Emerging Infections Research Resources Repository, BEI Resources: NR-9313 (HEK293T) and NR-2628 (MDCK); or from the Influenza Reagent Resource, IRR: FR-241 (HEK293T) and FR-242 (MDCK). It is important to keep track of the cell passage number, particularly for MDCK cells, since it can affect influenza B virus rescue efficiency and virus propagation. The use of late passage cells (above passage 50) is not recommended.
7. Viability of chick embryos can be determined before viral infection because live embryos move when candled. After viral infection, a dead egg will be easily identified by a small and bloody allantoic fluid volume.

8. A normal cell culture 5% CO_2 humidified incubator at 33 °C can be used to incubate infected eggs.
9. To accurately determine the concentration of the turkey or chicken RBCs, count the erythrocytes using a hemocytometer.
10. RNA extraction using TRIzol reagent (Invitrogen) is preferred when high titer or high concentration of virus is not available. Moreover, it is also recommended to use small volumes of water for the final reconstitution of RNA. Always use sterile, disposable plasticware and pipettes that are dedicated only to RNA handling, and RNase-free (ThermoFisher) or DEPC-treated ddH_2O.
11. Measure DNA and RNA concentrations at an absorbance of 260 nm. The 260/280 ratio is a good estimate of how pure is the sample is; a 1.8–2.0 ratio is optimal for RNA and DNA plasmid preparations. If the 260/280 ratio is lower, samples may be contaminated with proteins or other impurities.
12. Others available reverse transcriptase enzymes can also be used, following the manufacturer's recommendations.
13. For the amplification of other influenza B cDNAs, primers may be different.
14. When using another DNA polymerase, please refer to the respective manufacturer's recommendations.
15. For influenza B virus, the nucleotide sequences at the 3′ and 5′ ends of each vRNA segment are completely conserved [1, 58]. These noncoding region sequences, which are specific for each vRNA segment [1], represent the viral promoters for positive and negative RNA synthesis [1]. For influenza B virus, the last 10 nucleotides at the 3′ end and the last 9 nucleotides at the 5′ end are highly conserved, with the exception of position 6 (A/T) in the 3′ end [3, 32]. Thus, it is possible to amplify the entire genome of different influenza B viruses using universal primers.
16. BsmBI (CGTCTCN 1/5) belongs to type II restriction endonucleases that recognize asymmetric sequences and cleave these sequences at a defined distance. For cloning, use of this type II restriction endonuclease causes the respective restriction sites to disappear. This leaves the Pol-I promoter and terminator sequences to provide the exact 3′ and 5′ ends, respectively, of each vRNA that is required for efficient influenza B viral rescues.
17. BsmBI restriction sites are uncommon and therefore, not usually present in influenza B vRNA sequences. If an influenza B vRNA segment presents a BsmBI site, this can be still used for cloning into the pDP-2002. In this case, the viral cDNA should be first cloned into a shuttle vector (such as the pGEM-T vec-

tor series, Promega) and then subcloned into the pDP-2002 vector using partial digestion. If the BsmBI restriction site(s) are too close to the cDNA ends, they can be still cloned in the pDP-2002 vector by digestion with BsmBI using a multiple segment ligation protocol because the BsmBI site leaves four-nucleotide overhangs that will differ from each other. To avoid multiple cloning steps, it is possible to design a pair of universal primers for the specific influenza B vRNA segments to be cloned, in which the BsmBI cloning site is replaced with another restriction endonucleases (e.g., AarI, BbsI, or BsaI) that cuts outside its recognition site similarly to BsmBI.

18. Larger viral segments (e.g., PB2, PB1, and PA) are more difficult to amplify because of their length. If that is the case, consider using more cDNA as a template during the PCR reaction. Alternatively, use the initial PCR product in a second PCR reaction to amplify the full-length viral polymerase segments.
19. pDP-2002 contains the hRNA Pol-I promoter sequence. Due to the species specificity of the RNA Pol-I promoters, human cells are required for virus rescue. We recommend the use of HEK293T cells because of their high transfection efficiency for successful viral rescue [42]. Other reverse genetics systems based on promoters derived from other avian [59] or canine [60, 61] species have also been established for the rescue of influenza viruses.
20. Increased efficiency can be achieved adding more enzyme during the digestion at 55 °C. Thus, after 2 h incubation, add 1 μL of BsmBI and incubate the digestion mixture for an additional hour at 55 °C. Alternatively, incubate BsmBI digestions overnight for efficient DNA digestion.
21. pDP-2002 has an extra 444 nucleotides between the BsmBI restriction sites to verify BsmBI digestion. Thus, bands with a size of approximately 450 and 2950 nucleotides should be visible upon BsmBI digestion of pDP-2002. Contrary to the situation with pDP-2002, it is not possible to evaluate whether or not the influenza B cDNAs have been digested with BsmBI. Thus, the influenza B cDNAs can be first cloned into a shuttle vector such as pGEM-T (Promega) and subcloned into the pDP-2002 by BsmBI digestion.
22. Digestion of pDP-2002 with BsmBI does not leave compatible ends for religation. However, we recommend treating the BsmBI-digested pDP-2002 plasmid with SAP in case any pDP-2002 plasmids have been digested only at one of the BsmBI restriction sites.
23. Alternatively, the Rapid DNA ligation kit (Roche) can be used for ligation of pDP-2002 and the influenza B cDNAs.

24. Other *E. coli*-competent cells can be used for plasmid transformation.
25. Other media such as Luria-Broth (LB) or Super Optimal Broth (SOB) can be used to grow bacteria-competent cells during plasmids transformation.
26. pDP-2002 has ampicillin resistance. If another bidirectional plasmid is used for reverse genetics, complement with the appropriate antibiotic-containing LB liquid media and plates.
27. Plasmids containing influenza B glycoproteins (HA or NA) sometimes grow more efficiently at 33 °C.
28. If there are too many or too little bacteria colonies the next day, adjust the volume of the transformation to prepare new ampicillin LB plates.
29. Other miniprep or maxiprep purification kits can be used for isolation of plasmid DNA.
30. As indicated in **Note 16**, the use of type II restriction endonucleases results in the elimination of enzyme recognition sites (Fig. 2a). Therefore, BsmBI cannot be used to evaluate the presence of influenza B inserts in pDP-2002.
31. Cloning into pDP-2002 using BsmBI restriction sites results in the correct orientation of the influenza B cDNAs. Therefore, the orientation of the influenza B segments does not need to be evaluated.
32. Synthesis and amplification of influenza B vRNAs using RT-PCR can introduce undesired mutations and, therefore, the cloned cDNA will not be an accurate copy of the vRNA. To reduce the number of unwanted mutations, use high-fidelity RT and PCR kits and, always, sequence the entire cDNA to detect undesired mutations.
33. It is important to not cross-contaminate plasmid preparations. Carefully manipulate bacteria and plasmid DNA preparations to avoid unwanted bacteria and/or plasmid contaminations.
34. 34 Generate a glycerol stock of the bacteria cultures with the correct plasmids for long-term storage and to prevent further transformation of bacteria competent cells. A bacteria glycerol stock can be stored stably at −80 °C for years. In order to prepare the glycerol stock, add 500 μL of the overnight culture to 500 μL of 50% glycerol in a cryovial. Gently mix and store at −80 °C.
35. When other transfection reagents are used, follow the manufacturer's recommendations for optimal plasmid DNA transfection efficiencies.
36. Transfection in the absence of one of the pDP-2002 plasmids should be included as a negative control.

37. Alternatively to using co-cultures of HEK293T and MDCK cells, influenza B virus rescues can be achieved by transfecting HEK293T cells and passaging the tissue culture supernatants in fresh MDCK cells. Likewise, efficient influenza B virus rescues can be obtained by transfecting HEK293T cells and infecting 8–10-day-old chicken embryonated eggs directly with the transfected HEK293T cells or tissue culture supernatants.
38. Although less efficient than rescue in HEK293T cells, the hPol-I promoter also works in cells of primate origin and, therefore, virus rescue can be also achieved in Food and Drug Administration (FDA) vaccine-approved Vero cells [50, 62].
39. HEK293T cells detach more easily than MDCK cells. Tap the MDCK plates to help detachment in the 10 mm tissue culture plates.
40. Alternatively, cells can be resuspended in OptiMEM-I media.
41. We recommend transfecting approximately 10^6 HEK293T and MDCK cells. If necessary, count the cells using a hemocytometer and calculate the concentration of HEK293T and MDCK cells in the suspension to include 0.5×10^6 cells of each cell line per transfection.
42. Ratio of HEK293T-MDCK co-cultures is important for efficient influenza B viral rescues. We recommend a 1:1 ratio of HEK293T and MDCK cells. When cells are transfected in a monolayer, other HEK293T/MDCK co-culture ratios may be more appropriate (e.g., 3:1 or 2:1 ratio of HEK293T and MDCK cells).
43. Alternatively, use OptiMEM I media containing the TPCK-treated trypsin.
44. HEK293T/MDCK co-cultures can also be transfected while cultured in a monolayer. However, we have observed better transfection efficiencies when cells are transfected in suspension.
45. Usually, one confluent 100 mm dish of MDCK cells can be used to prepare 10–12 wells of a 6-well plate.
46. We recommend infecting an approximately 80–90% cell monolayer of MDCK cells to prevent TPCK-trypsin-mediated CPE.
47. We recommend making small volume aliquots (0.1–0.2 mL) to prevent multiple freeze-thaw cycles, which may reduce influenza B virus titers.
48. It is recommended to avoid breaking and collecting any of the egg's yolk. Collect only allantoic fluid. Some blood can be present in the collected allantoic fluid.
49. We recommend the use of turkey or chicken RBCs. Alternatively, other RBCs can be used to evaluate the presence of influenza B virus.

50. We recommend the use of immunofluorescence assays (8–10 h) over plaque (2–3 days) or $TCID_{50}$ (3–4 days) assays to first assess influenza B virus rescues and viral titers.
51. We recommend performing the IF and $TCID_{50}$ in triplicate and calculating viral titers with the triplicate average.
52. Influenza B virus infections greater than 8–10 h may lead to secondary infections and, therefore, result in an overestimation of viral titers.
53. Fixed and permeabilized cells can be stored at 4 °C in blocking solution for a longer period of time.
54. We recommend using an influenza B anti-NP monoclonal or polyclonal primary antibody because NP is one of the most abundant protein produced during viral infection. The nature (monoclonal or polyclonal), species specificity (mouse, rabbit, etc.), and proper dilution of the primary antibody should be previously evaluated. Several monoclonal and polyclonal antibodies against influenza B viruses can be obtained from BEI Resources (www.beiresources.org) and IRR (www.influenzareagentresource.org/).
55. Fluorescence will fade relatively quickly, especially with continuous exposure to light. Plates should be protected from light immediately after finishing the immunostaining procedure. Likewise, examination for an extended period of time under the fluorescent microscope will cause fading of fluorescence.
56. We recommend including a restriction site for an endonuclease to easily identify first, the recombinant nature of the rescued influenza B virus by enzymatic digestion. Positively identified virus clones can be sent for sequencing as confirmation.

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Chapter 15

Rescue of Infectious Salmon Anemia Virus (ISAV) from Cloned cDNA

Daniela Toro-Ascuy and Marcelo Cortez-San Martín

Abstract

The piscine Orthomyxovirus called Infectious Salmon Anemia Virus (ISAV) is one of the most important emerging pathogens affecting the salmon industry worldwide. The first reverse genetics system for ISAV, which allows the generation of recombinant ISA virus (rISAV), is an important tool for the characterization and study of this fish virus. The plasmid-based reverse genetics system for ISAV includes the use of a novel fish promoter, the Atlantic salmon internal transcribed spacer region 1 (ITS-1). The salmon, viral and mammalian genetic elements included in pSS-URG vectors allow the expression of the eight viral RNA segments. In addition to four cytomegalovirus (CMV)-based vectors that express the four proteins of the ISAV ribonucleoprotein complex, the eight pSS-URG vectors allowed the generation of infectious rISAV in salmon cells.

Key words Infectious Salmon Anemia Virus, ISAV, Viral RNA, Reverse genetics, Salmon cells, Transfection, ITS-1, RNA polymerase I and RNA polymerase II

1 Introduction

ISAV is a pleomorphic, enveloped virus with a diameter of 90–140 nm, in the family *Orthomyxoviridae* [1]. Its genome consists of eight RNA segments, single-stranded, of negative polarity((−)ssRNA) that encode at least ten proteins. The detailed study of other members of the family *Orthomyxoviridae,* such as Influenza virus A and B, has been possible thanks to the development of reverse genetics systems, which allow the manipulation of the viral genome [2]. The reverse genetics systems for influenza viruses are based on cloned cDNA copies of each segment into plasmids that permit transcription of the genomic viral RNAs from a RNA polymerase I promoter (pol-I) and viral protein expression from a RNA polymerase II promoter (pol-II), respectively (Fig. 1) [3–5]. An important obstacle in ISA virus rescue by reverse genetics has been the lack of an efficient promoter for RNA polymerase I, which was not previously described for the Atlantic salmon. As all the

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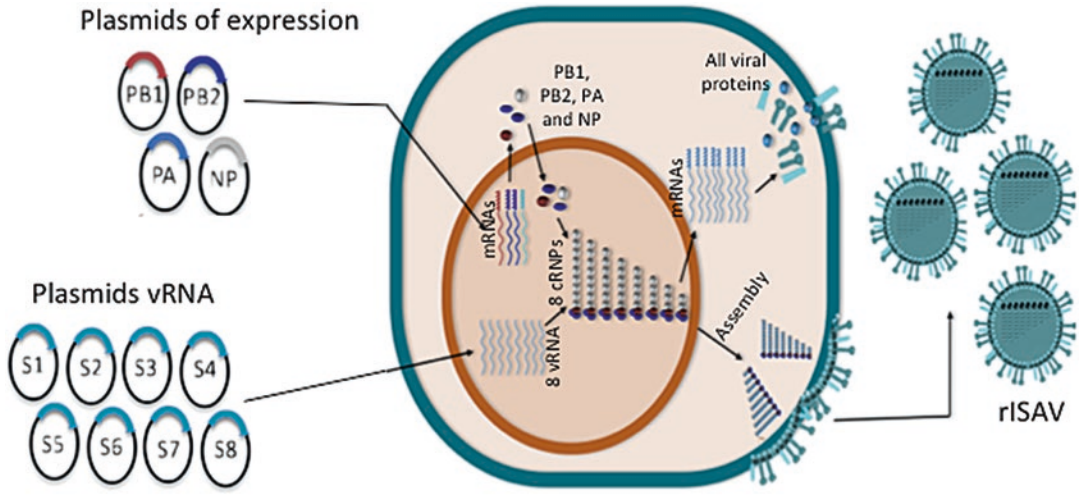

Fig. 1 Schematic diagram of the reverse genetics system for ISA virus rescue. ASK cells are co-transfected with 12 plasmids, 8 pSS-URG plasmids encoding each of the eight vRNA gene segments, and 4 plasmids expressing the viral polymerase complex and nucleoprotein (NP). After transcription of the vRNA segments in the nucleus by RNA polymerase I, they are associated with the polymerase complex and NP to form viral ribonucleoprotein (vRNP) complexes leading to de novo generation of ISA virus

promoters for RNA polymerase I are species specific [6], they do not have a clear genetic structure, and are found in the extensive intergenic spacer (IGS) region of ribosomal RNAs [7]. Identifying the sequences corresponding to the promoter for RNA polymerase I and its enhancers is difficult, considering that the IGS region in the *Salmo* genus varies between 15 and 23 kb in length [8, 9].

Understanding the virulence factors and pathogenic mechanisms of ISAV is essential to develop a better vaccine for it. Thus, we sought to develop a plasmid-based reverse genetics system to generate recombinant ISAV (rISAV) as a tool to further understand this virus. Due to the need of a salmon species-specific promoter with the characteristics of RNA polymerase I, we evaluated the use of internal transcribed spacer region 1 (ITS-1) comprised of 571 bp of *Salmo salar* [9]. The ITS1 of flatworms has been described to contain genetic elements such as a transcription promoter and regulator motifs with characteristics of functional ancestral sequences [10]. Recently, we showed that ITS-1 promoters drive the RNA synthesis by ISAV vRNA expression in a manner similar to that of an RNA polymerase I promoter [11]. In addition, to avoid the generation of vRNA molecules containing additional nucleotides in both RNA ends that affect the correct interaction between the viral genome and the viral polymerase, the hammerhead and the hepatitis δ virus ribozymes flank the 5′ and the 3′ end

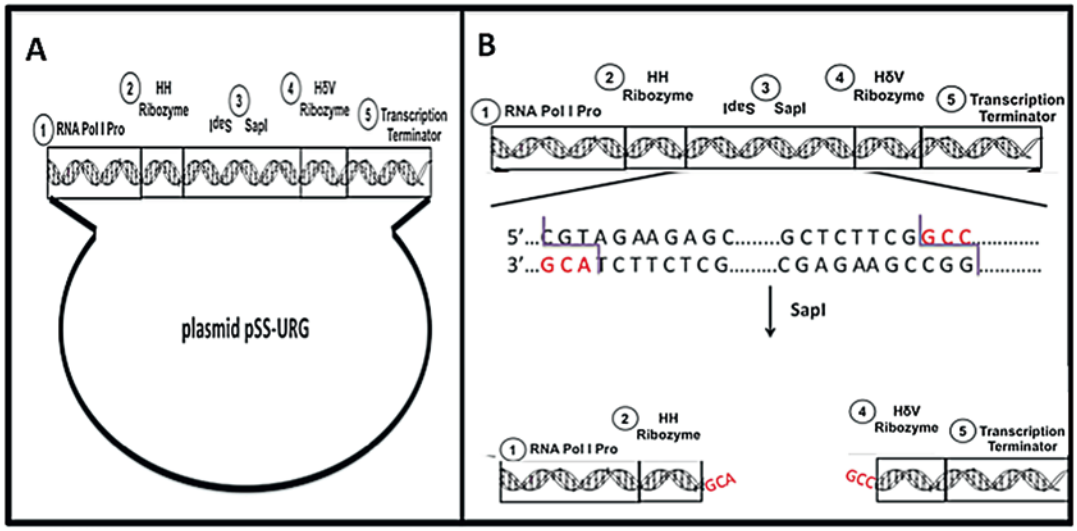

Fig. 2 Generation of the universal reverse genetics plasmid for ISAV. (**a**) Schematic of the design of the pSS-URG cassette. The universal vector contains the sequence of the *Salmo salar* promoter (RNA pol I pro), the hammerhead ribozyme (HH ribozyme), the ribozyme of the hepatitis δ virus (HδV ribozyme), and the transcription terminator of the rabbit β-globin (transcription Ter). (**b**) The schematic diagram illustrates the two SapI restriction site sequences in the pSS-URG, which is cut twice by *SapI*, yielding the vector fragments. The *SapI* sites in the vector are eliminated and the ISAV viral cDNAs can be inserted in only one orientation by T4 DNA ligase

of each vRNA, respectively (Fig. 2). Finally, to ensure the completion of transcription, the sequence of rabbit β-globin terminator was added to complete the design of the cassette [12]. With the strategic incorporation of all these elements into the plasmid system, the Atlantic salmon kidney (ASK) cells did not require any element *in trans* to generate recombinant ISAV. This system is capable of expressing intact genomic vRNA, allowing the full rescue of infectious rISAV.

2 Materials

2.1 ISAV Viral RNA Extraction

1. Commercial E.Z.N.A. Total RNA Kit I (Omega, Bio-Tek, Inc.). Add β-Mercaptoethanol to buffer TRK before use: 20 μL β-Mercaptoethanol per 1 mL buffer TRK.
2. "RNase Away" (Molecular Bio-Products, Inc., San Diego, CA, or Sigma-Aldrich, St. Louis, MO).
3. RNase-free 2 and 1.5 mL tubes.
4. Molecular biology grade ethanol (100%).
5. RNase-free, DEPC-treated water.
6. Microcentrifuge.

2.2 Viral cRNA Synthesis and PCR Amplification of Viral cDNAs

1. PCR Nucleotide Mix (dNTP; Promega).
2. SuperScript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA).
3. Primers for RT-PCR of each genomic segment for ISAV (Table 1).
4. RNase-free 0.2-mL PCR tubes.
5. RNase-free, DEPC-treated water.
6. Temperature/PCR cycler.
7. Analytic grade agarose (Lonza).
8. Wizard SV Gel and PCR Clean-Up System (Promega).

2.3 Cloning of Viral cDNAs into pSS-URG Vector

1. *SapI* (Thermo Scientific).
2. DNA T4 ligase (Thermo Scientific).
3. pSS-URG plasmid.
4. Temperature/PCR cycler.
5. Analytic grade agarose (Lonza).
6. Wizard SV Gel and PCR Clean-Up System (Promega).
7. RNase-free, DEPC-treated water.
8. Chemocompetent *Escherichia coli* Novablue cells (Novagen).
9. Luria-Bertani media (LB, MO BIO, Laboratories, Inc.).

Table 1
Primers used for the amplification of the eight genomic segments of ISAV via RT-PCR. The segment 1 was amplified in two fragment using both 1a and 1b pair primers.

	Primer F (forward)	Primer R (reverse)
Segment	5′-3′	5′-3′
1a	AGCTAAGAATGGACTTTATATCA GAAAACACG	AACCTTCGAAGCCAAACAGATAG
1b	CAATATCAAGTCCGTTCGAC GTGG	AGTAAAAAATGGACATTTTATTGATTAAA AGTATCGTC
2	AGCAAAGAACGCTCTTTAATAACC	AGTAAAAAATGCTCTTTTACTTATTAAAAAT
3	AGCAAAGATTGCTCAAATCCC	AGTTAAAATTGCTCTTTTCTTTATTTG
4	AGCTAAGATTGGCTGTTTCAAGA	AGTAAAAATTGGCTTTTTGGAAAA
5	AGTTAAAGATGGCTTTTCTAACA ATTTT	AGTAAAAATTGGCTATTTATACAATTAA TAATG
6	AGCAAAGATGGCACGATTCA	AGTAAAAAATGCACTTTTCTGTAAACG
7	AGCTAAGATTCTCCTTCTACAA TGGA	AGTAAAAATTCTCCTTTTCGTTTTAAA
8	AGCAAAGATTGGCTATCTACCA	AGTAAAAAAAGGCTTTTTATCTTTTG

10. Ampicilin 1 μg/mL (Sigma).
11. Pure Yield TM Plasmid Miniprep system (Promega).
12. Pure Yield TM Plasmid Midiprep system (Promega).

2.4 Generation of Protein Expression Plasmids

1. Protein expression vector pTriex-3 (Novagen) or pCI-neo (Promega).
2. Primers that amplify the PB2, PB1, PA, and NP ORFs of ISAV901_09 (Table 2).
3. Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA).
4. PCR Nucleotide Mix (dNTP; Promega).
5. RNase-free, DEPC-treated water.
6. Restriction enzymes NcoI and XhoI for ORF PB2 and PA, XhoI and SmaI for ORF PB1, MluI and XbaI for ORF of NP.
7. DNA T4 ligase (Thermo scientific).
8. Analytic grade agarose (Lonza).
9. Wizard SV Gel and PCR Clean-Up System (Promega).
10. RNase-free, DEPC-treated water.
11. Chemocompetent *Escherichia coli* Novablue cells (Novagen).
12. Luria-Bertani media (LB, MO BIO, Laboratories, Inc.)
13. Ampicilin 1 μg/mL (Sigma).
14. Pure Yield TM Plasmid Miniprep system (Promega).
15. Pure Yield TM Plasmid Midiprep system (Promega).

2.5 Transfection of Eukaryotic Cells with Plasmids for vRNA and Protein Synthesis

1. Regular growth medium: Leibovitz medium (L-15; HyClone), 10% Fetal bovine serum (Corning Cellgro, Mediatech), 50 μg/mL gentamicin (50 mg/mL, Hyclone), 40 μM β-mercaptoetanol (Gibco, Life Technologies), and 6 mM L-glutamine (200 mM, Corning Cellgro, Mediatech).

Table 2
Primers used for the amplification that amplify the PB2, PB1, PA, and NP ORFs of ISAV901_09 via RT-PCR

	Primer forward	Primer reverse
PB2	NcoI-ATGCCATGGACTTTATATCAGAAAA CACGATCAGCG	XhoI-CCGCTCGAGAACACCATATTCA TCCATAGG
PB1	SmaI-TCCCCCGGGAAACTCTAGTAGGTG	XhoI-CCGCTCGAGAACACGCTTTTTC TTCTTAATCAC
NP	MluI-CGACGCGTCATGGCCGATAAAGG TATGAC	XbaI-CGCTCTAGATCAAATGTCAGT GTCTTCCTC
PA	NcoI-CATGCCATGGATAACCTCCGT GAATGCATAAACC	XhoI-CCGCTCGAGTTGGGTACTGACT GCAATTTTC

2. Transfection medium: L-15 (HyClone), 40 μM β-mercaptoetanol (Gibco®, Life Technologies), and 6 mM L-glutamine (200 mM, Corning Cellgro, Mediatech).
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
4. 12-well plates (Nunc).
5. Fugene 6 (Promega).
6. Atlantic salmon kidney cells (ASK, ATCC_CRL-2747).
7. pSS-URG/S1-S8 plasmids and protein expression plasmids (PB2, PB1, PA, and NP).
8. Incubator at 18 °C.

2.6 Amplification of ISA Viruses

1. Infection medium: Leibovitz medium (L-15; HyClone), 50 μg/mL gentamicin (50 mg/mL, Hyclone), 40 μM β-mercaptoetanol (Gibco, Life Technologies), and 6 mM L-glutamine (200 mM, Corning Cellgro, Mediatech).
2. Regular growth medium.
3. Incubator at 18 °C.
4. Atlantic salmon kidney cells (ASK, ATCC_CRL-2747).
5. 6- or 12-well plates (Nunc).
6. PBS.

3 Methods

The generation of ISAV viruses can be divided into the following steps: (1) Extraction and amplification of the ISAV viral RNAs. (2) Sequence analysis of the viral RNAs. (3) Cloning of the viral cDNAs into the appropriate plasmid vectors for vRNA (pSS-URG) or protein synthesis. (4) Transfection of cells with reverse genetics plasmids. For efficient ISAV generation, it is essential to use cell lines susceptible to replication of ISAV (i.e., ASK cells), and to optimize the parameters of transfection in this cell line. (5) Amplification of generated viruses.

3.1 ISAV Viral RNA Extraction

1. Mix 350 μL of virus stock with 350 mL of buffer TRK. The protocol outlined here is based on the E.Z.N.A. total RNA kit I; however, other RNA extraction kits or procedures may be used (*see* **Note 1**).
2. Add cold 700 μL of EtOH 70% in sterile DEPC-treated water.
3. Transfer lysed virus solution onto a HiBind RNA column placed in a 2-mL collection tube.
4. Centrifuge at 10,000 × *g* for 1 min at room temperature.
5. Wash the HiBind RNA column with 500 μL of RNA Wash buffer I.

6. Centrifuge at 10,000 × *g* for 1 min at room temperature, and discard the flow-through.
7. Add 500 µL of RNA Wash buffer II directly into the HiBind RNA column.
8. Centrifuge at 10,000 × *g* for 1 min at room temperature, and discard the flow-through.
9. Repeat **steps 7** and **8**.
10. Centrifuge the HiBind RNA column at maximum speed for 2 min to completely dry the HiBind RNA column.
11. Pipette 40 µL of RNase-free water onto membrane.
12. Centrifuge for 1 min at 10,000 × *g* to elute RNA.
13. Store RNA at −80 °C until further use.

3.2 Viral cRNA Synthesis and PCR Amplification of Viral cDNAs

The following protocol is based on SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase. Other commercially available enzymes or kits for reverse transcription and PCR may be used.

1. Mix the following components on ice:

Extracted RNA	8 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
dNTPs (10 mM)	1.5 µL
RNase-free water	1 µL

2. Incubate at 65 °C for 5 min.
3. Immediately place tube on ice.
4. Add the following components:

2× buffer (provided with enzyme)	12.5 µL
SuperScript III RT/Platinum Taq mix (Invitrogen)	1 µL
(Total volume: 25 µL)	

5. RT-PCR amplification:

RT cycle	50 °C for 30 min
One cycle	94 °C for 2 min
35 cycles	94 °C for 15 s 52 °C (segments 1 and 6) or 49 °C (segments 2–5 and 7–8) at 30 s 68 °C at 2 min and 15 s (segments 1–4) or 1 min and 30 s (segments 5–8)
One cycle	68 °C for 5 min

6. Visualize the PCR products in a 0.8% agarose gel (w/v), and then purified from the gel with the Wizard SV Gel and PCR Clean-Up System according to the manufacturer's instructions.
7. Sequence the PCR products obtained for each genomic segment of ISAV.

3.3 Cloning of Viral cDNAs into pSS-URG Vector

The eight genomic segments of ISAV901_09 were synthesized by Genscript Co in the cloning vector pUC57 with each of its ends containing a SapI restriction site.

For cloning of viral cDNAs into pSS-URG vector (*see* **Note 2**), digest with restriction enzyme SapI (*see* **Note 3**).

1. 2 μg of each pUC57 vector containing the genomic segments ISAV (cDNA) with the restriction enzyme SapI (5 U enzyme/μg of DNA).
2. 1 μg of vector pSS-URG with the restriction enzyme SapI (5 U enzyme/μg of DNA).
3. Incubate digestion for 2 h at 37 °C.
4. Visualize the digestion products in a 0.8% agarose gel (w/v), and then purify from the gel with the Wizard SV Gel and PCR Clean-Up System according to the manufacturer's instructions.
5. Ligate the digested fragments using the following ligation mix:

   1 μL of DNA ligase-T4 (5 U/μL).

   Ratio 5:1 (insert: linear plasmid).

   1 μL Buffer 10× DNA ligase T4.

   RNase-free water (Total volume: 10 μL).
6. Incubate for 16 h at 16 °C.
7. Use 10 μL of ligation mix to transform 100 μL of chemically competent *E. coli* Novablue cells.
8. Incubate for 30 min on ice, 45 s at 42 °C and 5 min on ice.
9. Add 900 μL of medium LB.
10. Incubate for 1 h at 37 °C with agitation.
11. Plate the bacterial cells on LB-agar with 1 μg/mL ampicillin.
12. Incubate for 16 h at 37 °C.
13. Analyze 20 bacterial clones for rapid analysis of colonies [13].
14. Purify positive clones using the Pure Yield TM Plasmid Miniprep system according to the manufacturer's instructions.
15. Sequence recombinant plasmids for each segment using primers specific for the Hδ and HH ribozyme sequences.
16. Purify positive clones (without spurious mutations by sequencing) using Pure Yield TM Plasmid Midiprep system according to the manufacturer's instructions.

3.4 Generation of Protein Expression Plasmids

1. In addition to the eight pSS-URG plasmids for the transcription of the eight ISAV viral RNA segments, plasmids for the expression of the ISAV viral proteins PB2, PB1, PA, and NP must be prepared.
2. Design oligonucleotides that amplify the PB2, PB1, PA, and NP open-reading frames.
3. As a template for PCR amplification, use the respective (a) viral cDNAs or (b) pSS-URG plasmids.
4. Clone PCR products into a protein expression vector, such as pTriex3 or pCI-neo.
5. Sequence the resulting constructs to confirm the absence of unwanted mutations introduced by PCR.

3.5 Transfection of Eukaryotic Cells with Plasmids for vRNA and Protein Synthesis

1. Seed 2.5×10^4 ASK cells/cm^2 in a 12-well plate.
2. Incubate cells for 72 h at 18 °C in regular growth medium. Cells should be 70% confluent at the time of transfection.
3. Premix DNAs for transfection (9 μg total): Use 250 ng each of the PB2, PB1, PA, and NP protein expression plasmids and 1 μg each of the eight pSS-URG plasmids for the transcription of ISAV viral RNA. Total premix DNA volume should ≤100 μL.
4. Transfection cocktail:
   (a) Add 94 μL of transfection medium to an Eppendorf tube.
   (b) Add 6 μL of transfection reagent (Fugene 6) per premix of DNA.
   (c) Incubate for ~5 min at room temperature.
   (d) Add premixed DNAs.
   (e) Vortex for 1 min and spin down.
   (f) Incubate transfection mixture for 30 min at room temperature.
5. Transfection:
   (a) Growth medium needs to be aspirated and the cells need to be washed.
   (b) Wash cells twice with PBS; add 1 mL of PBS.
   (c) Remove PBS and add transfection cocktail dropwise to cells.
   (d) After adding transfection mixture, add 300 μL of additional transfection medium dropwise.
6. Incubate for 4 h at 18 °C.
7. Remove supernatant and add 1 mL of growth medium.
8. Controls should include the following:
   (a) Untreated ASK cells.
   (b) ASK cells treated with transfection reagent to monitor cytotoxic effects of the transfection reagent.

(c) ASK cells transfected with plasmids for vRNA synthesis, but not plasmids for protein synthesis; no virus should be recovered from this control.

(d) ASK cells transfected with plasmids for protein synthesis, but not plasmids for vRNA synthesis; no virus should be recovered from this control.

9. Incubate cells for 7 days at 16 °C; typically, no cytopathic effect (CPE) is observed (*see* **Note 4**).
10. Collect virus-containing supernatant from transfected cells.
11. Optional:
    (a) Spin down supernatant for 5 min at room temperature to pellet ASK cells.
    (b) Transfer supernatant to fresh tube.
12. Store virus-containing supernatant at −80 °C until further use (ideally store sample in aliquots).

3.6 Amplification of ISA Viruses

1. Seed 2.5×10^4 ASK cells/cm^2 in a 6 or 12-well plate in regular growth medium.
2. Incubate cells for 3 days at 18 °C. Cells should be 80–90% confluent at the time of infection.
3. Prepare tenfold dilutions of virus-containing supernatant collected from transfected ASK cells. Dilute supernatant in infection medium.
4. Wash ASK cells twice with PBS.
5. Add 400 or 800 μL of (un)diluted virus-containing supernatant to ASK cells. Infect ASK cells for 4 h at 16 °C.
6. Wash ASK cells three times with PBS to remove virus inoculum.
7. Incubate cells with regular growth medium.
8. Observe cells daily for CPE:
    (a) CPE is indicative of virus replication.
    (b) For undiluted virus-containing supernatant, CPE typically appears within 7 days of infection.
    (c) If no CPE is observed, analyze sample by RT-PCR in supernatant (*see* **Note 5**)
9. When ~80% of ASK cells are lysed, harvest virus-containing supernatant.
10. Spin down supernatant for 5 min at 4 °C to pellet floating cells.
11. Transfer virus-containing supernatant to fresh tube.
12. Store virus-containing supernatant at −80 °C until further use (ideally store samples in aliquots).

13. Before the virus is used for further studies, its sequence should be confirmed.
14. Before the virus is used for further studies, virus stocks should be grown, and their titers should be determined by plaque assay [14].

4 Notes

1. A minimum of 1 mL of supernatant is required for RNA extraction and RT-PCR performed on the 8 genome segments.
2. The pSS-URG vector was designed to incorporate any genomic segment of any ISAV strain.
3. The incorporation of a restriction site for *SapI* allows nucleotide-specific fusion of any two DNA segments without the introduction of mutations or unwanted nucleotides.
4. If the virus cannot be rescued, or the rescue efficiency is low, the following modifications can be attempted:
   (a) Test different amounts and/or different ratios of protein expression plasmids.
   (b) Test different amounts and/or ratios of different transfection reagents.
   (c) Incubate transfected cells for 10–14 days.
5. Use qRT-PCR to quantify the vRNA from segment 8 to determine the copy numbers from all of the cell culture supernatants [15].

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Chapter 16

Plasmid-Based Reverse Genetics of Influenza A Virus

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Abstract

Influenza A viruses have broad host range with a recognized natural reservoir in wild aquatic birds. From this reservoir, novel strains occasionally emerge with the potential to establish stable lineages in other avian and mammalian species, including humans. Understanding the molecular changes that allow influenza A viruses to change host range is essential to better assess their animal and public health risks. Reverse genetics systems have transformed the ability to manipulate and study negative strand RNA viruses. In the particular case of influenza A viruses, plasmid-based reverse genetics approaches have allowed for a better understanding of, among others, virulence, transmission, mechanisms of antiviral resistance, and the development of alternative vaccines and vaccination strategies. In this chapter we describe the cloning of cDNA copies of viral RNA segments derived from a type A influenza virus into reverse genetics plasmid vectors and the experimental procedures for the successful generation of recombinant influenza A viruses.

Key words Influenza A virus, Plasmid-based reverse genetics, Virus rescue approaches, Recombinant influenza A virus, Bidirectional plasmids

1 Introduction

Influenza viruses are single stranded, negative-sense RNA viruses with a segmented genome within the family *Orthomyxoviridae*, which includes seven genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Influenzavirus D, Thogotovirus, Isavirus, and Quaranjavirus [1]. Influenza A virus (IAV) contains eight viral gene segments, which encode at least 12 viral proteins (Fig. 1). Two surface proteins, the trimeric hemagglutinin (HA) and the tetrameric neuraminidase (NA), are the main antigenic determinants of the virus to which neutralizing antibodies are elicited. IAVs are classified by the antigenic properties of the HA and NA. Currently, 18 HA and 11 NA subtypes have been described [2, 3]. Wild aquatic birds are considered the primary hosts for IAVs in which 16 HA and 9 NA subtypes have been found (Fig. 2). HA subtypes 17 and 18 in combination with NA subtypes 10 and

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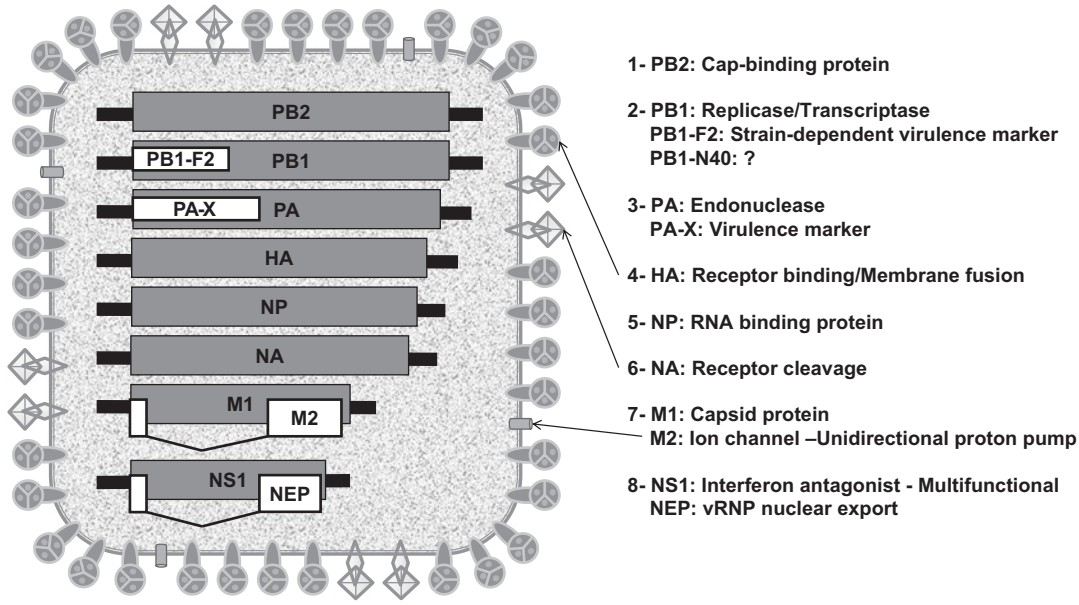

Fig. 1 Influenza A virion structure. Type A influenza viruses are enveloped viruses that carry eight RNA segments of negative polarity in the form of vRNPs and protected by the matrix protein (M1). Each segment encodes at least one open reading frame. Segment order (1–8) and encoded genes for each segment are shown. *Arrows* indicate cartoon representations of HA (homotrimer), NA (homotetramer), and M2 (homotetramer) on the virus surface. A "*?*" indicates unknown biological significance. "Virulence marker" is used to indicate effects in virulence in experimental in vitro and/or in vivo models which can be either strain and/or host specific

11, respectively, have been identified in fruit bats, but not yet in birds [2–4]. In aquatic birds the majority of influenza A viruses replicate in the cells lining the intestinal tract, cause no apparent disease, and are transmitted by fecal contamination of water [5, 6]. These viruses occasionally establish stable lineages in land-based birds and a limited number of mammalian species including horses, pigs, seals, and humans, usually associated with respiratory illness [7]. Segment exchange between influenza A viruses during infection and replication provides a mechanism to increase both the genetic diversity and potentially the host range of these viruses. Human influenza pandemic strains have an origin in the avian reservoir either from a direct avian source, as in the 1918 Spanish influenza pandemic [8], or through intermediary hosts, such as pigs, as in the 2009 pandemic [9]. Typically, IAVs from one animal species must undergo some change, either in the form of specific amino acid changes and/or reassortment before they can cross species barriers. Understanding influenza host switching is very complex and represents a monumental task; however, reverse genetics has allowed for important advances with respect to understanding molecular changes associated with cross-species transmission of avian influenza A viruses in mammals [10–13].

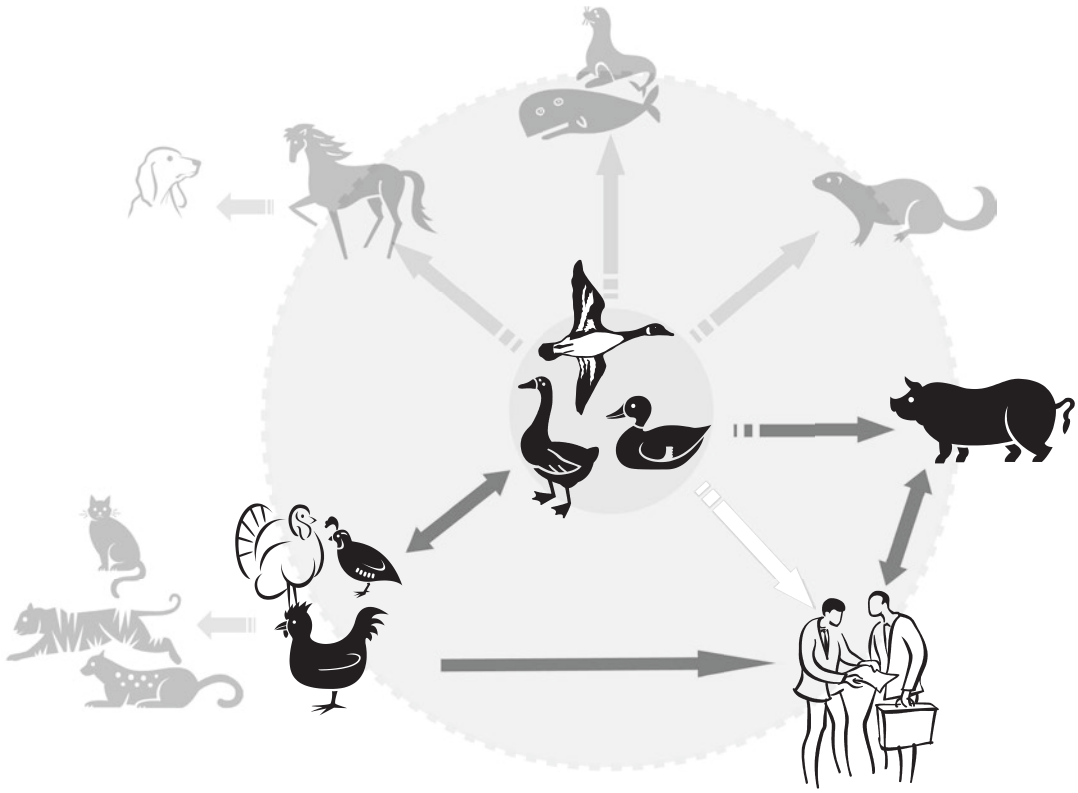

Fig. 2 Influenza A virus wheel. Wild aquatic birds of the world are considered the natural reservoir of all influenza viruses. There is extensive evidence for transmission of influenza viruses between wild ducks and other species. Two-way transmission events between pigs and humans have been extensively documented and led to the emergence of the 2009 H1N1 pandemic strain

Reverse genetic systems for RNA viruses have been available for many years [14]. For reverse genetics of influenza viruses (Fig. 3), transcription of the mRNA for the viral proteins is typically done from a plasmid encoding a RNA polymerase II (pol II) promoter element upstream of cloned viral cDNA, and expressed in a manner similar to host mRNA [15]. Synthesis of the negative-sense, single-stranded, uncapped vRNAs can be accomplished by the host's RNA polymerase I (pol I) [15]. In eukaryotic cells, the RNA pol I complex produces uncapped ribosomal RNA. Transcription is terminated with the (murine) RNA polymerase I terminator (T-1), also known as Sal I box, to produce vRNAs with defined start and stop sites, respectively. An alternative method to produce vRNAs is through the use of a T7 RNA polymerase promoter directly upstream of viral cDNA cloned in the negative sense [16]. The 3′ end of the vRNA is formed by the hepatitis delta ribozyme cleavage sequenced cloned immediately downstream. Transcription of the eight vRNAs, together with the four protein expression plasmids responsible for viral replication and

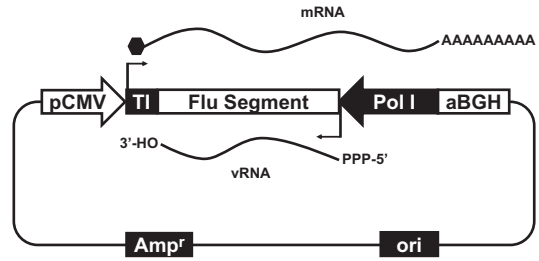

Fig. 3 Generation of influenza A virus by plasmid-based reverse genetics. Typical reverse genetics systems for influenza viruses require the cloning of cDNA copies of each vRNA segment into suitable vectors. The figure depicts one of the most versatile cloning vectors with bidirectional promoters [20]. Rescue of influenza virus de novo requires the production of vRNAs and viral proteins (particularly RNA polymerase subunits and NP) from host derived DNA-dependent RNA polymerase complexes. The expression of vRNAs requires promoters with exact 5′ initiation and 3′ stop sites, like the human RNA polymerase I promoter (Pol I). Viral protein synthesis is achieved with the use of RNA polymerase II promoters (pCMV or others)

transcription (PB2, PB1, PA, and NP), allows for the generation of influenza in 293T and a variety of other human derived cell types [17, 18]. Bidirectional vectors have been made that contain pol II and pol I promoters in opposite directions to drive expression of both mRNA and vRNA species from the same plasmid, thus consolidating the viral rescue system into eight plasmids [19]. In this chapter, we describe the use of reverse genetic techniques for type A influenza virus using the 8-plasmid system with bidirectional promoters as originally described by Hoffman et al. [19]. Minor variations in the procedures compared to those described in the previous chapter are included to teach the reader on the flexibility of the rescue systems. In addition, we provide description on alternative reverse genetics vectors based on the swine polymerase I (swpol I) and chicken (avian) polymerase I (ckpol I) promoters. Background information regarding each procedure has been kept to a minimum because it is extensively covered in the previous chapter. Please also note that downstream procedures for analysis and characterization of influenza A and B viruses are the same, therefore they are not covered in this chapter.

2 Materials

2.1 Influenza A Viruses

Work with type A influenza viruses should follow biosafety laboratory (BSL) rules and regulations appropriate for this type of work. This protocol is described for the rescue of type A influenza strains that require BSL-2 conditions. This protocol does not cover additional biosafety requirements and risk assessment analysis of those

strains considered select agents and/or that require BSL-3 or higher conditions for work. All material used for the rescue of influenza A viruses should be sterilized before disposal (*see* **Note 1**).

2.2 Laboratory Equipment

Certified biosafety cabinets, tissue culture incubators, egg incubators, low to medium speed swing-bucket centrifuge, swing-bucket rotors and adapters, microcentrifuge with a rotor capable of reaching up to 12,000 × *g*, thermocyclers, heat blocks, water baths, spectrophotometer (Nanodrop or similar), agarose electrophoresis chamber, cell counter, tissue culture media and supplies and molecular biology plasticware and supplies (*see* **Note 2**).

2.3 Influenza A vRNA Extraction

RNA is prone to degradation if proper handling techniques are not followed. Precautions for handling RNA samples include purchasing reagents that are free of RNases, separating reagents and materials (tips, tubes, ddH_2O, etc.) for RNA work only, and wearing sterile gloves.

1. RNeasy Mini Kit (Qiagen).
2. ß-ME (beta mercaptoethanol). ß-ME is toxic and therefore the container must be opened in a fume hood.
3. 200 proof ethanol (for molecular biology).
4. RNAse-free H_2O or DEPC-treated ddH_2O.

2.4 cDNA Synthesis

1. Universal (Uni)-12 primer (5′-AGC AAA AGC AGG-3′).
2. Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Promega).
3. RNasin 40 U/μL (Promega).
4. 2.5 mM dNTPs (Thermo Fisher).
5. RNAse-free H_2O or DEPC-treated ddH_2O.

2.5 Amplification of Influenza A vRNAs

1. Influenza A virus primer set for cloning into pDP2002 (Table 1).
2. Expand high fidelity polymerase (Roche) or
3. Phusion high fidelity polymerase (NEB).
4. 50× dNTPs (20 mM Thermo Fisher).
5. RNAse-free (ThermoFisher) or DEPC-treated ddH_2O.
6. Agarose LE, analytical grade for molecular biology applications (Promega).
7. Gel and DNA purification kits [such as QIAquick Gel Extraction Kit and MinElute PCR purification Kit (Qiagen)].

2.6 Cloning Influenza A cDNAs

1. BsmBI, BsaI, AarI, BbsI restriction enzymes (NEB).
2. Quick T4 DNA ligase (NEB).

Table 1
Oligonucleotides for cDNA synthesis, PCR amplification, cloning, and sequencing of influenza A virus reverse genetics system

Reverse transcription				
	Uni12	AGCAAAAGCAGG		
pDP2002 cloning				(*see* **Note 16**)
Segment	Forward primer		Reverse primer	
PB2	Bm-PB2 1F	TATTCGTCTCAGGG**AGCGAAAGCAGG**TC	Bm-PB2 2341R	ATATCGTCTCGTATT**AGTAGAAACAAGG**TCGTTT
PB1	Bm-PB1 1F	TATTCGTCTCAGGG**AGCGAAAGCAGG**CA	Bm-PB1 2341R	ATATCGTCTCGTATT**AGTAGAAACAAGG**CATTT
PA	Bm-PA 1F	TATTCGTCTCAGGG**AGCGAAAGCAGG**TAC	Bm-PA 2233R	ATATCGTCTCGTATT**AGTAGAAACAAGG**TACTT
HA	Bm-HA 1F	TATTCGTCTCAGGG**AGCAAAAGCAGG**GG	*Bm-NS 890R*	
NP	Bm-NP 1F	TATTCGTCTCAGGG**AGCAAAAGCAGG**GTA	Bm-NP 1565R	ATATCGTCTCGTATT**AGTAGAAACAAGG**GTATTTTT
NA	Bm-NA 1F	TATTCGTCTCAGGG**AGCAAAAGCAGG**AGT	Bm-NA 1413R	ATATCGTCTCGTATT**AGTAGAAACAAGG**AGTTTTTT
M	Bm-M 1F	TATTCGTCTCAGGG**AGCAAAAGCAGG**TAG	Bm-M 1027R	ATATCGTCTCGTATT**AGTAGAAACAAGGT**AGTTTTT
NS	Bm-NS 1F	TATTCGTCTCAGGG**AGCAAAAGCAGG**GTG	Bm-NS 890R	ATATCGTCTCGTATT**AGTAGAAACAAGG**GTGTTTT
Restriction site adapter variations (pDP2002)				
	BsaI forward	TATTGGTCTCAGGG**AGC…**	BsaI reverse	ATATGGTCTCGTATT**AGT…**
	AarI forward	TATTCACCTGCTGCAGGG**AGC…**	AarI reverse	ATATCACCTGCTGCATATT**AGT…**
	BbsI forward	TATTGAAGACGAGGG**AGC…**	BbsI reverse	ATATGAAGACGATATT**AGT…**
Alternative reverse genetic vectors				
Segment	pMACk2009 reverse primers		pPIG2012 reverse primers	
PB2	BmCk-PB2 2341R	ATATCGTCTCGCTGT**AGTAGAAACAA GG**TCGTTT	BmPIG-PB2 2341R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**TCGTTT
PB1	BmCk-PB1 2341R	ATATCGTCTCGCTGT**AGTAGAAACAA GG**CATTT	BmPIG-PB1 2341R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**CATTT
PA	BmCk-PA 2233R	ATATCGTCTCGCTGT**AGTAGAAACAA GG**TACTT	BmPIG-PA 2233R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**TACTT
HA	*BmCk-NS 890R*		*BmPIG-NS 890R*	

NP	BmCk-NP 1565R	ATATGGTCTCGCTGT**AGTAGAAACAAGG**GTATTTTT	BmPIG-NP 1565R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**GTATTTTT
NA	BmCk-NA 1413R	ATATCGTCTCGCTGT**AGTAGAAACAAGG**AGTTTTTT	BmPIG-NA 1413R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**AGTTTTTT
M	BmCk-M 1027R	ATATCGTCTCGCTGT**AGTAGAAACAAGG**TAGTTTTT	BmPIG-M 1027R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**TAGTTTTT
NS	BmCk-NS 890R	ATATCGTCTCGCTGT**AGTAGAAACAAGG**GTGTTTT	BmPIG-NS 890R	ATATCGTCTCGAGAT**AGTAGAAACAAGG**GTGTTTT
Alternate restriction site adapters				
	pMACk2009 reverse adapters		pMACk2009 reverse adapters	
	BsaI reverse	ATATGGTCTCGCTGT**AGT…**	BsaI reverse	ATATGGTCTCGAGAT**AGT…**
	AarI reverse	ATATCACCTGCTGCACTGT**AGT…**	AarI reverse	ATATCACCTGCTGCAAGAT**AGT…**
	BbsI reverse	ATATGAAGACGACTGT**AGT…**	BbsI reverse	ATATGAAGACGAAGAT**AGT…**
pDP2002 colony PCR screening				
	Seq T7	TAATACGACTCACTATAGG		
	Seq PolI	GAGGTATATCTTTCGCTCCG		

3. Top10 or DH10B chemically competent cells (Invitrogen).
4. Luria-Broth (LB) liquid media.
5. Super Optimal broth with Catabolite repression (SOC) media.
6. LB agar plates containing 50 μg/mL Ampicillin.
7. Ampicillin 1000× (Sigma).
8. QIAquick Gel Extraction Kit and MinElute PCR purification Kit (Qiagen).
9. HiSpeed Plasmid Maxi kit (Qiagen).
10. Agarose LE, analytical grade for molecular biology applications (Promega).

2.7 Bidirectional Reverse Genetics Plasmid Vectors

Plasmid pDP2002 (Fig. 4) is a derivative of pHW2000 [19] in which a 444 nt spacer sequence was incorporated between the two BsmBI sites in order to monitor and more efficiently obtain double-digested vector for the purpose of cloning full length influenza A cDNAs. The pDP2002 plasmid contains two transcription units in opposite orientations. The first unit contains the human polymerase I promoter (hpol I) and the murine polymerase I transcription terminator (T-1) for the synthesis of vRNAs. The second unit drives viral mRNA transcription from the polymerase II-driven cytomegalovirus promoter (pCMV) and the bovine growth hormone polyadenylation signal (aBGH). The use of bidirectional

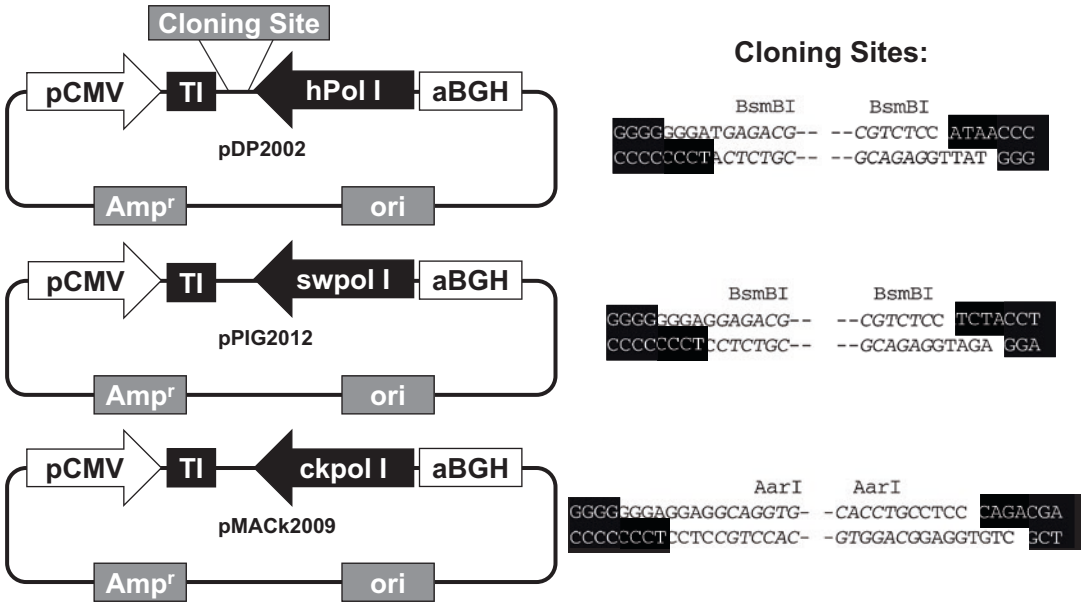

Fig. 4 Plasmid vectors for reverse genetics. Reverse genetic vectors vary with respect to the species of origin of the RNA polymerase I promoter and the sequence of the cloning site into which cDNA segments of influenza are ligated

promoter plasmids reduces the number of reverse genetics vectors to 8 (*see* **Note 3**). Influenza A viral cDNAs are cloned into the reverse genetics vectors using restrictions sites artificially engineered at the 5′ and 3′ ends of each segment in a manner compatible with the reverse genetics vector.

Plasmids are prepared using commercially available plasmid preparation kits, following the manufacturer's recommendations, and are best stored at −20 °C at a concentration of 0.25–1.0 mg/mL. We recommend that plasmid stocks are diluted into working stocks at a concentration of 100 ng/μL prior to transfection to minimize pipetting errors and to standardize pipetting volumes for each plasmid.

2.8 Cell Lines for the De Novo Generation of Influenza A Viruses

Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection, ATCC, CRL-11268). Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34). Low passage MDCK cells are recommended. For growth of influenza A viruses, MDCK cells at passage number of ≤50 are recommended. Both cell lines are maintained in appropriate tissue culture media at 37 °C in a 5% CO_2 tissue culture incubator (*see* **Note 4**).

Alternative cell lines for de novo generation of influenza virus (Subheading 3.7) with compatible plasmid systems: *Sus scrofa* (pig) epithelial kidney cells PK(15) (ATCC, CCL-33) and *Gallus gallus* (chicken) embryo fibroblasts DF-1cells (ATCC, CRL-12203), maintained in appropriate tissue culture media at 37 °C in a 5% CO_2 tissue culture incubator.

2.9 Tissue Culture Media and Reagents

1. T-75 tissue culture flasks, canted neck (Corning).
2. 6-, 12-well tissue culture plates (Corning).
3. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (1×) liquid; with L-glutamine; without sodium pyruvate (Invitrogen).
4. Opti-MEM I Reduced Serum Medium (1× Invitrogen).
5. 100× Antibiotic-Antimycotic solution (AB, Sigma).
6. HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) Buffer (1 M stock solution, Invitrogen).
7. Phosphate buffered saline 10× concentrate, BioPerformance Certified, suitable for cell culture (Sigma).
8. Bovine albumin fraction V (7.5%, Invitrogen)
9. Heat inactivated (56 °C, 30 min) fetal bovine serum (FBS), 40 nm filtered (HyClone Laboratories, Inc.). Aliquot prior to use.
10. Trypsin-EDTA (Trypsin-ethylenediaminetetraacetic acid, 0.05% trypsin; 0.53 mM EDTA.4Na, Sigma).
11. Trypsin L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK-trypsin); treated (type XIII from bovine pancreas, Sigma).

12. Transit-LT1 transfection reagent (Mirus).
13. Allantoic fluid from uninfected 9-day-old SPF embryonated chicken eggs (Subheading 3.7), used as supplement for tissue culture media, may be collected in advance and stored in single-use aliquots at −80 °C.

2.9.1 Media Preparations

1. Complete DMEM: 470 mL DMEM, 5 mL AB, 12.5 mL Bovine albumin fraction V (7.5%), 12.5 mL HEPES buffer.
2. Preparation of TPCK-trypsin stock solution (1 mg/mL, 1000×): Dissolve 10 mg TPCK-trypsin in 10 mL Complete DMEM or 1× PBS (Sigma). Filter through 0.2 μm membrane. Store in single-use 100 μL aliquots at −20 °C.
3. Medium for cell growth: Complete DMEM with 5–10% (v/v) FBS
4. Medium for virus propagation: To 500 mL Complete DMEM or Opti-MEM, add 0.5 mL TPCK-trypsin stock solution.
5. PBS 1×: Dilute 1:10 PBS 10× with ddH2O. Sterilize by autoclaving Store at room temperature (*see* **Note 5**).

3 Methods

3.1 Extraction of Influenza A vRNA

RNAses are ribonucleases that catalyze the degradation of RNA and are commonly found on work surfaces and skin, particularly on fingertips [14]. Disposable gloves must be worn at all times while handling RNA samples. Dedicated equipment, supplies and reagents solely for RNA work is highly recommended. RNA samples are to be maintained on ice throughout the entire RNA extraction procedure and, preferably, be used immediately for cDNA synthesis. Remaining RNA samples can be kept frozen at −70 °C. Typically RNA samples stored for more than a month are discarded.

The vRNAs are extracted using silica-based (RNeasy Mini Kit, Qiagen) or organic-based (TRIzol reagent, Invitrogen) reagents or any other available RNA extraction method following procedures recommended by the manufacturer (*see* **Note 6**). The procedure below uses the RNeasy mini kit, which has provided consistent and reliable results of influenza A vRNA with adequate purity and devoid of contaminants and RNAses. Viral titers of $\geq 10^6$ $TCID_{50}$ ($\geq 10^7$ EID_{50}) are typically required for efficient preparation of vRNAs and subsequent RT-PCR amplification. Virus isolation, from clinical samples, in tissue cultured MDCK cells or 8–10-day-old chicken embryonated eggs are recommended in order to obtain appropriate quantities of vRNA for downstream steps. Extracted vRNAs are eluted and resuspended in RNase-free ddH_2O. RNA concentration is quantified using a NanoDrop or similar spectrophotometer.

3.1.1 Procedure Using Qiagen Minicolumns

1. Prepare ß-ME/RLT buffer solution: 10 µL ß-ME/mL in RLT buffer.
2. To 200 µL of tissue culture supernatant (TCS) or allantoic fluid (AF) in 1.5 mL microcentrifuge tube.
3. Add 350 µL ß-ME/RLT solution.
4. Mix by pipetting up and down several times.
5. Add 550 µL 70% ethanol.
6. Mix by pipetting up and down several times.
7. Transfer 750 µL of mix from the previous step to an RNeasy mini column (mini column should be already in a collection tube).
8. Centrifuge 1 min @ max speed in microfuge.
9. Discard flow-through from collection tube. Return column to collection tube.
10. Repeat with remaining volume of the RNA mix.
11. Add 700 µL of RW1 buffer into mini column.
12. Centrifuge 1 min @ max speed in microfuge.
13. Discard collection tube. Put mini column in a new collection tube.
14. Add 500 µL of RPE buffer into spin column.
15. Centrifuge 1 min @ max speed in microfuge.
16. Discard flow-through from collection tube.
17. Add 500 µL of RPE buffer into spin column.
18. Centrifuge 1 min @ max speed in microfuge.
19. Discard flow-through from collection tube.
20. Return mini column to collection tube.
21. Centrifuge 1 min @ max speed in microfuge to dry RNA.
22. Transfer mini column to 1.5 mL Microcentrifuge tube.
23. Add 40 µL RNAse-free H_2O. Incubate 1 min @ RT.
24. Centrifuge 1 min @ max speed in microfuge to collect RNA.

3.2 cDNA Synthesis Using Purified Influenza A vRNA

3.2.1 In a 1.5 mL Microcentrifuge Tube Add

1. RNA (from Subheading 3.1)	4.0 µL
2. Uni12 primer (0.1 µg/µL)	0.5 µL
3. RNAse-free H2O	5.5 µL

4. Heat sample at 70 °C for 5 min.
5. Incubate on ice immediately for 2 min.
6. Spin down in microcentrifuge to collect contents (<1 min).
7. Put tube back on ice.

3.2.2 In a Separate 1.5 mL Microcentrifuge Tube Prepare the Rt Cocktail by Adding the Following Components (Adjust Volume Based on Total Number of cDNA Preparations to Be Performed)

8. 2.5 mM dNTPs (Thermo Fisher)	4.0 µL
9. 5× Reverse Transcriptase Buffer	4.0 µL
10. Rnasin 40 U/µL (Promega)	1.0 µL
11. AMV Reverse Transcriptase (Promega)	1.0 µL

12. Mix well. Add RT cocktail to RNA sample.
13. Incubate @ 42 °C for 1–2 h.
14. Heat inactivate @ 70 °C for 10 min (*see* **Note 6**).

3.3 PCR Amplification of Full-Length Influenza A Viral cDNAs

For full-length amplification of influenza A virus gene segments from cDNA copies, reliable results are obtained using either the Expand High Fidelity PCR system (Roche) or Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB). Please *see* Table 1 for a list of segment specific primers. Forward and reverse primers contain sequences complementary to the viral segment and 5′ extensions encoding either BsmBI, BsaI, AarI, or BbsI sites for cloning purposes (*see* **Notes 6** and 7).

In a PCR tube add the following:

1. cDNA	1.0 µL
2. Primer 1—forward (100 ng/µL)	1.5 µL
3. Primer 2—reverse (100 ng/µL)	1.5 µL

3.3.1 In a Separate Microcentrifuge Tube Add the Following to Prepare the Amplification Mixture (Adjust Volume Accordingly Based on Number of cDNA Samples to Be Amplified)

4. 50× dNTPs (20 mM, Thermo Fisher)	1.0 µL
5. 10× Expand HF Buffer (Roche)	5.0 µL
6. Expand HF or Phusion (high GC)	0.75 µL
7. ddH_2O	39.25 µL

Add amplification mixture to cDNA/primer pair sample. Use proper sterile techniques to not cross-contaminate samples. Set PCR machine with the following parameters: 94 °C 4 min, (94 °C 20 s, 56 °C 30 s, 72 °C 7 min*) × 30 cycles, 72 °C 10 min, 4 °C ∞ and run PCR. *7 min for P and HA genes; 5 min for NP, NA, M, and NS genes.

Analyze and purify PCR products by standard 1% agarose gel electrophoresis. Purification of PCR reaction products from agarose gels can be performed using a variety of gel extraction kits. After elution, PCR products can be quantified using a NanoDrop or similar spectrophotometer. PCR reactions and purified PCR products can be stored at −20 °C until further use (*see* **Notes 6–8**).

3.4 Cloning Influenza A Viral cDNAs into pDP2002

From the step above, eight PCR products are expected to contain full-length cDNA copies of influenza A virus segments flanked by appropriate restriction sites. The resulting PCR products will be

used to clone into the corresponding reverse genetics vector. Following gel extraction (using QIAquick gel extraction kit or similar), the PCR product is eluted in 27 μL elution buffer (Qiagen) or ddH_2O.

3.4.1 Restriction Enzyme Digestion of PCR Products

1. Eluted PCR product	26.0 μL
2. Restriction Enzyme Buffer 10×	3.0 μL
3. BsmBI, BsaI, AarI, or BbsI	1.0 μL

Digestion can be performed for 4 h to overnight in a PCR machine, heat block or water bath set at the appropriate temperature following manufacturer's recommendations. If using a water bath, please note potential excessive evaporation resulting from prolonged incubation periods; spinning down contents frequently prevents restriction digestion issues. The digested fragment is purified using QIAquick PCR purification kit (use minelute columns). Digested product is eluted using 10 μL of elution buffer (Qiagen). Gel purification of PCR product after digestion is not necessary.

3.4.2 Restriction Enzyme Digestion of Vector pDP2002

1. pDP2002 (~100 ng/μL)	8.0 μL
2. Buffer 3 10× (NEB)	6.0 μL
3. BsmBI (NEB)	2.0 μL (≤1 μL should be sufficient)
4. ddH_2O	44.0 μL

Incubate at 55 °C, 3 h to overnight in a PCR machine or heating block. Run digested products by electrophoresis in 1% agarose gel, the large fragment is purified using QIAquick Gel extraction kit. It should be very obvious on the gel the presence of two major bands, one of ~0.4 kb corresponding to the spacer region and one of ~2.9 kb corresponding to the vector. If the vector has been digested at a single BsmBI site, a band of ~3.4 kb should be seen. Only the 2.9 kb band should be used for cloning. The vector is eluted in 30 μL EB (Qiagen). Please note that although it is common practice to use ethidium bromide while running the gel, such practice may lead to mutations in the vector resulting in defective clones. It is best to run the gel without ethidium bromide followed by incubation of the gel for 15 min in ethidium bromide solution (0.5 μg/mL) once the run has been completed.

3.4.3 Ligation and Transformation

1. Digested PCR product (~180 ng or ddH_2O negative control)	5.0 μL
2. Digested pDP2002 (~40 ng)	1.5 μL
3. 2× Quick ligase buffer	7.5 μL
4. Quick T4 DNA ligase (New England Biolabs)	1.0 μL

Incubate at room temperature for 5 min, then immediately put on ice. The ligation reaction can be kept at −20 °C. During the 5 min incubation time, remove one tube of *E. coli* Top10 competent cells from −70 °C and thaw on ice. Also allow the SOC medium to reach room temperature, if it is maintained frozen or refrigerated.

To a sterile screw cap 1.5 centrifuge tube, add 25 μL competent cells, and then 2 μL of ligation reaction, mix well by stirring the tip (do not pipette up and down to mix because it affects the viability of chemically competent *E. coli* cells). Incubate on ice for 30 min.

Transform *E. coli* cells by heat shock incubating Top10 cells/ligation suspension at 42 °C for 30 s, then immediately bring back the tube on ice for 2 min.

Add 250 μL SOC medium to the bacterial cells and incubate at 37 °C for 45 min to 1 h in a shaker incubator (200 rpm). Addition of SOC medium can be performed inside a Biosafety Cabinet or near an open flame following appropriate microbiology practices.

While the cells are incubated in the shaker, have LB/Ampicillin agar plates (containing 50 μg/mL Ampicillin) ready. If the LB agar plates have been kept at 4 °C, it is possible that they may have accumulated excessive moisture on its surface, which can later affect plating of bacterial cells. To avoid such problem, LB agar plates can be placed inside a biosafety cabinet with the lids open in order to let them air dry.

Finally, using a sterile "hockey stick" spreader, spread entire SOC/cells/ligation suspension on the LB/Ampicillin plate. Place plate in the 37 °C incubator for 16–18 h with the agar side up and the lid side down. The next day, colonies should be visible to the naked eye containing potentially positive clones. A colony PCR reaction can be performed using appropriate set of primers to screen for positive clones (Table 1) (*see* **Notes 9** and **10**).

3.5 Colony PCR Screening for Positive RG Clones

Since it is expected for the user to pick several colonies from one or more plates, it is best to prepare a PCR master mix with a volume appropriate for the number of colonies to be screened. Please ignore from the calculation the volume corresponding to the Taq polymerase and the volume corresponding to the colony itself. When preparing a large master mix (≥5 reactions), mix by vortexing 10 s at medium speed and centrifuge briefly to spin down contents to the bottom of the tube. Maintain master mix on ice. Distribute, in pre-chilled PCR tubes, 20 μL of PCR master mix/reaction. Add Taq polymerase last, and maintain tubes on ice until ready for PCR reaction.

3.5.1 Master Mix

1. Seq PolI (100 ng/μL)	0.6 μL
2. Seq T7 (100 ng/μL)	0.6 μL
3. 50× dNTPs (20 mM)	0.4 μL
4. 10× Buffer A (Fisher)	2.0 μL
5. Taq polymerase (Fisher)	0.25 μL
6. ddH_2O	16.5 μL

3.5.2 Identification of Positive Colonies

There are at least four alternatives for picking up colonies (around 1.5–3 mm in diameter) using a sterile tip or sterile toothpick

1. Directly pipet into the PCR cocktail, or
2. Pipette up and down into a 1.5 mL tube containing 10 μL LB/Amp and then use ~0.2 μL for PCR. Keep 1.5 mL tube at 4 °C to later amplify positive clones, or
3. Shake (do not pipet up or down) tip into 50 mL Falcon tube containing 4–5 mL LB/Amp. Use what remains attached on the tip to pipet up and down into a tube containing the PCR cocktail. Keep Falcon tube at 4 °C to later amplify positive clones, or
4. Make a replicate of the colony into another plate (place a grid underneath to later identify the positive clones) and use what remains attached on the tip to pipet up and down into a tube containing the PCR cocktail. Incubate grid plate at 37 °C to let colonies grow again.

   Finally, pipet colony up and down (from one of the four alternatives above) into PCR tubes containing the 20 μL PCR cocktail, vortex briefly, centrifuge to spin down contents and bring back to ice. Set PCR machine with the following parameters: 94 °C 5 min, (94 °C 30 s, 55 °C 30 s, 72 °C 5 min) × 30, 72 °C 10 min, 4 °C ∞. Run PCR.

   Once PCR reaction is complete, load 5 μL PCR product on a 1% agarose gel with ethidium bromide (0.2–0.5 μg/mL) to monitor for presence of clones carrying fragments of expected size. In addition to or instead of colony PCR, positive colonies can also be screened by mini-prep DNA preparation and restriction digestion. A virtual plasmid map of the expected clone, if sequences are known, facilitates the interpretation of screening methods. Regardless of the screening method, positive clones must be sequenced in order to rule out the presence of spurious mutations in either the influenza gene segment or plasmid vector (particularly on promoter regions).

Once the appropriate influenza A reverse genetics plasmid set has been obtained, high-quality plasmid DNA preparations for mammalian transfection are needed. It is recommended the use of endonuclease-free maxiprep DNA kits (Qiagen) following the manufacturer's directions. Plasmid DNA concentrations are determined using a NanoDrop or similar spectrophotometer. Typical pDNA concentrations range from 0.25 to 1.0 mg/mL. We recommend keeping plasmid DNA stocks at −70 °C and preparing working solutions at 100 ng/μL of each plasmid in order to preserve consistency and to avoid pipetting errors. It is also recommended that prior to each transfection, the integrity of plasmid DNAs be evaluated by agarose gel electrophoresis (*see* **Notes 11** and **12**).

3.6 Generation of Influenza A Virus by Reverse Genetics

The following procedure has been routinely called "Easy Flu" by Erich Hoffman to highlight the simplicity and efficiency with which influenza A viruses can be recovered by reverse genetics using the 8-plasmid system. Using this procedure it is also very easy to cross-contaminate transfection reactions if not careful. Use extreme care when handling cells, transfection reagents, plasmids, and media. The procedure below is described for performing transfections using a 6-well plate format and cocultured HEK293T/MDCK cells. It is also amenable for scaling down to 12- and 24-well plate formats. And although it is theoretically amenable also for smaller well plate sizes, the smaller the well, the fewer the number of cells susceptible for transfection and the less efficient the recovery of influenza viruses becomes (*see* **Notes 13** and **14**).

HEK293T cells and MDCK cells are routinely grown in T75 flasks and maintained as mentioned above in DMEM complete culture media. Prior to working with cells, pre-warm PBS and cell culture media at 37 °C.

3.6.1 Day 1: In a Biosafety Cabinet and at 24 h Prior to Transfection, Freshly Confluent HEK293t and MDCK Cells Are Subjected to the Following

1. Washed once or twice with 5 mL 1× PBS or 1× medium without FBS.
2. Incubated with 10 mL trypsin-EDTA (20–45 min for MDCK cells, 1 min for 293T).
3. Cells are removed from the flask using disposable pipettes (with filter), placed in 15 mL conical Falcon tubes, spun down for ~5 min at low speed (800 rpm) in swing bucket centrifuge.
4. Supernatant is discarded by gently removing it with a serological pipet.
5. Cells are resuspended in 10 mL Opti MEM I, supplemented with 1× Antibiotic-Antimycotic solution (Opti MEM I-AB).
6. Remove 1 mL of each cell line and mix into 18 mL Opti MEM I-AB and mix well. Tube will now contain ~20 mL of cell suspension. For both experienced and inexperienced

investigators, it is best to count cells using a hemocytometer prior to seeding on the plate. A HEK293T/MDCK cell ratio of 1:1–5:1 can be used. Consistent virus rescue results are obtained when HEK293T cells are seeded at 4.5×10^5 cells/well and MDCK cells are seeded at 1.0×10^5 cells/well in this cocultured format (4.5:1 ratio).

7. Add the cell suspension to a 6-well plate, 3 mL per well.

3.6.2 Day 2: The Next Day (16–24 h After Cell Seeding), Prepare the Cocktail of Eight Plasmids (One Plasmid per Influenza Viral RNA Gene Segment) in a 1.5 mL Microcentrifuge Tube. Please Note That the Use of Filter Tips Is Recommended for This and Subsequent Steps to Avoid Potential Contaminations

1. If a working stock of each plasmid has been prepared at 100 ng/μL, pipet 10 μL of each plasmid (1 μg of each plasmid, 8 μg pDNA).
2. To the plasmid DNA cocktail, add 20 μL of *Opti MEM I (1:2.5 ratio)*. Mix well. The tube will now contain ~100 μL.
3. In a separate tube prepare 85 μL of *Opti MEM I* and add 16 μL of TransIT-LT1 (2 μL/μg of plasmid DNA). The tube will now contain ~100 μL. *It is easier to calculate the total amount of Opti MEM I/*TransIT-LT1 *needed for all the transfections and prepare a cocktail with the total volume (plus extra ~10% excess for both).*
4. Vortex the Opti MEM I/TransIT-LT1 cocktail and add 100 μL to the plasmid DNA/Opti MEM I mixture. Mix well. *It is essential to change tips to go to the next sample, the system is so sensitive that cross-contaminations will occur if proper care is not taken when handling the samples.*
5. Incubate the mixture at room temperature for approximately 45 min, and then add 800 μL of OptiMEM I-AB.
6. Remove the medium from the cells in the 6-wells plate, add the transfection mixture to the cells ($t = 0$). Incubate the cells for 6–12 h in a 5% CO_2 incubator at 37 °C. Washing the cells prior to addition of the transfection mixture is not necessary. Use caution when adding transfection mixture onto the cells. It is best to place the pipet tip as close as possible to the cell monolayer and pipet down dropwise while at the same time preventing the formation of aerosols.
7. After 6–12 h transfection, remove the transfection mixture from cells (very slowly), add 1 mL of OptiMEM I-AB to the cells and incubate at 37 °C until 24 h post-transfection (hpt) in a 5% CO_2 incubator. Removal of transfection reaction is best accomplished by tilting the plate slightly towards the user, inserting a pipet tip touching the wall of the well most proximal to the user and pipetting up slowly until most of the medium has been removed. It is not crucial to wash the cells after this step before going into next.
8. At 24 hpt, add 2 mL of OptiMEM I-AB containing 1.5 μg/mL TPCK-trypsin to the cells (1 μg/mL final concentration).

3.6.3 Day 3 Onwards (48–72 h Post-transfection): Monitor Cells for Cytopathic Effect (CPE), Particularly in the MDCK Cells (See **Notes 13** *and* **14***). Small Foci of Dead Cells Indicative of Active Influenza A Virus Replication may Become Apparent for Some, But Not All, Successful Virus Rescues. Regardless of the Presence of CPE, at 72 hpt (Earlier and/or Later if Desired) Proceed by*

1. Withdrawing 1 mL of the cell culture supernatant to a microcentrifuge tube and centrifuge at 5000 rpm (~1300 × *g*) for 5 min.
2. Remove 900 μL of the supernatant to a new fresh tube and add 500 μL/well to MDCK cells for a blind passage (i.e., 12-well plate).
3. Incubate the supernatant with MDCK cells for 1 h at room temperature, discard the supernatant and add virus growth medium containing 1 μg/mL of TPCK-trypsin and monitor for the presence of virus by CPE and/or HA assay at 48–72 hpi (as described in Chapter 11).

CPE and/or HA positive samples indicative of influenza A virus rescue can be either stored at −70 °C for later or further amplified in either MDCK cells or 8–10-day-old chicken embryonated eggs in order to produce virus stocks. Details on virus amplification and characterization are extensively covered in Chapter 11 (*see* **Note 15**).

3.7 Generation of Influenza A Virus by Reverse Genetics Using Alternative Ambisense Plasmid Vectors

While efficient, the generation of influenza A viruses by a pol I approach is thought to be species specific requiring a species match between the pol I promoter and the cell type [15]. Herein, we describe the procedure to produce influenza A viruses in porcine cell and avian cell types using species specific bidirectional vectors, pPIG2012 and pMACk2009, respectively.

The pPIG2012 vector (Fig. 4) is a derivative of pHW2000 in which the 212 nt of the human pol I promoter sequence was replaced with a 168 nt sequence corresponding to the swine pol I promoter. The pPIG2012, like pHW2000, contains two BsmBI sites for cloning of full-length cDNA copies of influenza A virus gene segments. Viral segments are amplified and cloned into pPIG2012 in essentially the same manner as described in Hoffmann et al. [11, 16] and before with alternative reverse primers. The pMACk2009 is a derivative of pDP2002 in which the human pol I promoter sequence was replaced with a 415 nt sequence corresponding to the chicken pol I promoter (Fig. 4). A spacer region of 29 nt flanked by AarI sites is removed and replaced with the corresponding full-length cDNA copies of influenza A virus gene segments. Materials and procedures for cloning of influenza segments into either pPIG2012 or pMACk2009 are the same as for pDP2002, except that treatment of digested vectors with Shrimp Alkaline Phosphatase is recommended to minimize background noise from the vectors' self-ligation.

Influenza A virus reverse genetics rescue using the pPIG2012-based vector is carried out using PK(15) cells, which are routinely maintained in DMEM supplemented with 10% FBS, 25 mM HEPES, and 1× antibiotic-antimycotic solution. For virus rescue, PK(15) cells are cocultured with MDCK cells at a ratio of ~4:1. In a 6-well plates 4×10^5 PK(15) cells and 1.0×10^5 MDCK cells are seeded per well. Transfection conditions, precautions, and downstream procedures are as explained above (Subheading 3.6).

For influenza virus rescue using the pMACk2009, consistent results are obtained using DF-1 cells. This cells are maintained in DMEM supplemented with 10% FBS, 25 mM HEPES, and 1× antibiotic-antimycotic solution. Since DF-1 cells are sensitive to the action of TPCK-trypsin, the reverse genetics procedure is modified from the one described above (Subheading 3.6) as follows:

3.7.1 Day 1: DF-1 Cells Are Seeded at 1.5–1.8 × 10^6 Cells per Well in a 6-Well Seeded 24 h Prior Transfection. The Use of Cocultured MDCK Cells Is Omitted

1. Incubate the mixture at room temperature for 45 min, and then add 800 μL of OptiMEM I-AB/0.15% BSA.
2. Remove the medium from the cells in the six-well plate, add the transfection mixture to the cells ($t = 0$). Incubate the cells at 37 °C for 6 h in a 5% CO_2 incubator. Washing the cells prior to addition of the transfection mixture is not necessary.
3. At 6 hours post-transfection, remove the transfection mixture from cells (very slowly) and replace with Opti-MEM AB supplemented with 0.15% BSA and 5% allantoic fluid from 9-day-old embryonated chicken eggs. The allantoic fluid serves as a source of trypsin-like proteases necessary for influenza A virus growth that at the same time helps DF-1 cells to remain attached to the well.

3.7.2 Day 2: Plasmid DNA/Opti-MEM I and Opti-MEM I/Transit LT1 Is Prepared as Described in Subheading 3.6, Except that Opti-MEM Is Supplemented with 0.15% (v/v) of BSA (Bovine Albumin Fraction V, 7.5%)

3.7.3 Day 3 Onwards (48–72 hpt)

1. Remove 1 mL of the cell culture supernatant to a microcentrifuge tube and centrifuge at 5000 rpm for 5 min.
2. Remove 900 μL of the supernatant to a new tube and add 500 μL/well to MDCK cells for a blind passage (i.e., 12-well plate).
3. Incubate the supernatant with the MDCK cells for 1 h, discard the supernatant and add virus growth medium containing 1 μg/mL of TPCK-trypsin and monitor for the presence of virus by CPE and/or HA assay at 48–72 hpi (as described in Chapter 11).

CPE and/or HA positive samples indicative of influenza A virus rescue can be either stored at −70 °C for later or further amplified in either MDCK cells or 8–10-day-old chicken embryonated eggs in order to produce virus stocks.

4 Notes

1. Work with influenza A viruses must be conducted under appropriate biosafety (BSL) conditions. Most institutions require inspections and approvals of laboratories prior to conducting work with infectious disease agents. Prior to engaging in reverse genetics studies with influenza A viruses, consult with

pertinent biosafety officials on the type of biocontainment required at your institution.

2. Generation of aerosols while working with influenza A viruses must be prevented. Adequately maintain and inspect equipment dedicated to work with influenza A viruses.
3. Other ambisense/bidirectional plasmids, such as pDZ [17] or pHW2000 [11], can be used for reverse genetics of influenza A viruses with appropriate amplification primers.
4. HEK293T and MDCK cells are also available from the Biodefense and Emerging Infections Research Resources Repository, BEI Resources: NR-9313 (HEK293T) and NR-2628 (MDCK); or from the Influenza Reagent Resource, IRR: FR-241 (HEK293T) and FR-242 (MDCK). It is important to keep track of cell passage number, particularly for MDCK cells, since it affects influenza A virus growth. The use of late passage cells (above passage 50) is not recommended.
5. Tissue culture media, supplies and reagents and alternative transfection reagents are available through different vendors. Listed above are those that have provided optimal results in our hands.
6. Other available RNA extraction reagents and methods can also be used, following the manufacturer's recommendations. RNA extraction using TRIzol reagent (Invitrogen) is preferred when high titer or high concentration of virus is not available. Synthesis of cDNA can be performed using other reverse transcriptases following manufacturer's directions. The PCR amplification step is crucial; the use of other thermophilic DNA polymerase enzymes is possible but those mentioned above have provided the most robust results.
7. Primers can be modified based on specific needs. In influenza A viruses, the first 12 nucleotides at the 3′ end and last 13 nucleotides at the 5′ end of each vRNA segment are highly conserved and the basis for the design of universal (Uni) primers [18–20].
8. Larger viral segments (e.g., PB2, PB1, and PA) are more difficult to amplify because of their length (>2 kb). Consider using more cDNA as a template during the PCR reaction. Alternatively, performing PCR amplification of overlapping fragments of smaller size followed by a second overlapping PCR yields satisfactory results.
9. It is not uncommon to find PCR fragments that are difficult to clone into any of the reverse genetics vectors. A combination of inefficient restriction enzyme digestion and other inherent properties of the PCR product may in part explain such failures. If that is the case, consider other PCR cloning shuttle vectors and proceed with subcloning into the bidirectional rescue plasmid after a suitable candidate has been obtained. For cloning unstable inserts, consider the use of other *E. coli* strains for improved stability. Alternatively, decreasing temperature

(e.g., 30 °C) helps with the cloning of unstable inserts into the ambisense rescue plasmids.

10. Other ligation methods can be used following the manufacturer's recommendations.
11. Digestion of pDP2002 with BsmBI does not leave compatible ends for self-ligation. However, carry over vector digested at a single site may lead to background noise in the absence of inserts. Consider the use of alkaline phosphatases that catalyze the dephosphorylation of 5′ and 3′ ends of DNA (heat labile Shrimp Alkaline Phosphatase, for example) in order to reduce background. We recommend the use of positive (digested plasmid vector plus a well characterized cloning fragment) and negative control ligation reactions (digested plasmid vector without PCR fragment), in order to determine background noise levels. A ≥10:1 positive to background ratio is expected under normal conditions.
12. Other *E. coli* competent cells prepared by chemical method or electroporation can be used for plasmid transformation. LB liquid media, SOC liquid media, LB agar plates, and Ampicillin 1000× can be purchased from commercial vendors ready to use or can be prepared in house.
13. High quality plasmid preparations devoid of protein and other impurities are crucial for influenza A virus reverse genetics. Measure DNA and RNA concentrations at an absorbance of 260 nm using a Nanodrop or similar spectrophotometer. The 260/280 ratio is used to estimate sample purity. A plasmid preparation with a 260/280 nm ratio of ≥1.8 is highly recommended. Other miniprep or maxiprep purification kits can be used for isolation of plasmid DNA.
14. HEK293T cells may detach in the presence of TPCK-trypsin and absence of serum. This is not indicative of virus rescue.
15. While procedures for analysis and characterization of influenza A and B viruses are the same, these viruses have distinct optimal growth temperatures. In tissue culture, influenza A viruses grow at 35–37 °C and influenza B viruses replicate ideally at 33–35 °C. In eggs, the recommended temperature for growth of influenza A viruses is 35 °C while influenza B viruses are grown at 33 °C.
16. While cloning adapters are described with BsmBI restriction sites, BsaI, AarI or BbsI restriction sites may be used. Restriction sites should be chosen in such a way that internal digestion of the segment should be avoided. Bm-NA 1F and Bm-NA 1413R will amplify NA segments from N1, N2, N4, N5, and N8 subtypes. pMACk2009 and pPIG2012 utilize the universal forward primers used for pDP2002. Alternative restriction sites may also be used on the reverse primers for pMACk2009 and pPIG2012, however care must be taken in their design to include the appropriate overhangs for each vector.

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