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Insertion of a GFP Reporter Gene in Influenza Virus

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Abstract

The incorporation of a fluorescent reporter gene into a replication competent influenza A virus (IAV) has made it possible to trace IAV infection *in vivo*. This protocol describes the process of inserting a green fluorescent protein (GFP) reporter into the IAV genome using the established reverse genetics system. The strategy begins with the reorganization of segment eight of the IAV genome, during which the open reading frames of non-structural protein 1 (NS1) and the nuclear export protein (NEP) are separated to allow for GFP fusion to the NS1 protein. The NS1, GFP, and NEP open reading frames (ORF) are then cloned into the IAV rescue system backbone. Upon construction of the GFP encoding segment eight rescue plasmid, recombinant NS1-GFP influenza virus can be rescued via co-transfection with the remaining seven rescue plasmids. The generated NS1-GFP IAV can subsequently be used to visualize infected cells both *in vitro* and *in vivo*.

Keywords

Influenza A virus; recombinant virus; reverse genetics; GFP reporter; nonstructural protein 1 (NS1)

INTRODUCTION

The existence of an efficient and effective reverse genetics system for influenza A virus (IAV) allows for the study of viral pathogenesis and virus host interactions through the generation of recombinant and reassortant viruses (Basler and Aguilar, 2008; Fodor et al., 1999; Manicassamy et al., 2010; Neumann et al., 1999; Pappas et al., 2008; Quinlivan et al., 2005). As IAV requires the encoded RNA dependent RNA polymerase for transcription and replication, both negative sense genome and positive sense transcripts must be expressed in cells in order to rescue viable virus. To this end, a bidirectional plasmid system (pDZ, (Quinlivan et al., 2005)) has been designed that incorporates both an RNA Polymerase I promoter and an RNA Polymerase II promoter flanking each influenza gene, such that the negative sense is expressed in cells via the RNA Polymerase I promoter, and viral mRNA is expressed in cells via the RNA polymerase II promoter (Figure 1). Insertions, deletions, or modifications can be made in these rescue plasmids and used for the generation of recombinant IAV. This protocol discusses the use of this established IAV rescue system for construction of a green fluorescent protein (GFP) reporter influenza virus (Manicassamy et al., 2010).

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In order to generate an IAV carrying a fluorescent marker, we utilized the smallest segment (segment eight, NS) and fused the GFP gene to the major protein product, nonstructural protein 1 (NS1). To ensure proper production of the second protein product, the nuclear export protein (NEP), the splice acceptor site was removed and the NEP open reading frame (ORF) was duplicated and inserted behind a porcine teschovirus-1 2A site, such that NEP would be translated independently of the NS1-GFP fusion protein (Figure 2A). Once the segment eight genome is reorganized and the GFP gene inserted in-frame with NS1 within the rescue system, recombinant virus can be rescued using tissue culture based methods. The resulting virus will express NS1-GFP during viral infection, thus GFP will serve as a marker for infected cells and can be used to quantify levels of viral replication.

This unit describes the generation of a recombinant IAV strain carrying the GFP gene in the eighth segment of the viral genome. Basic Protocol 1 describes the construction of the NS1-GFP fusion within the pDZ rescue plasmid. Basic Protocol 2 discusses the rescue of viable NS1-GFP IAV using tissue culture based methodologies.

BASIC PROTOCOL 1

Construction of NS segment containing GFP reporter

This protocol describes the generation of an IAV segment eight (NS) rescue plasmid encoding for an NS1-GFP fusion protein through the reorganization of the NS genome. The bidirectional rescue plasmid (pDZ) serves as the backbone for this insertion, such that the newly engineered NS segment can be combined with the other seven pDZ rescue plasmids for transfections and subsequent IAV rescues (Hoffmann et al., 2000; Quinlivan et al., 2005). This approach can be applied to any strain of IAV for which a rescue system exists; here we discuss the manipulation of the A/Puerto Rico/8/1934 NS segment for GFP insertion as an example.

As the NS segment encodes for two proteins through the utilization of splicing, insertion of GFP into the NS1 ORF can only be achieved after the separation of the NS1/NEP ORFs by sequence duplication (Figure 2A). In this way the GFP ORF can be inserted at the C terminal tail of NS1 in place of the stop codon without perturbing the NEP ORF. A small peptide linker (GSG) is also included between the NS1 and GFP ORFs to provide flexibility and enable NS1 functionality. To allow for proper NEP expression from the NS segment, the splice acceptor site is removed, and the 19 amino acid Porcine teschovirus-1 ribosome stop/start 2A site is incorporated between the GFP and NEP ORFs (Figure 2B and C) (Donnelly et al., 2001). This will result in the synthesis of a single poly-transcript for NS1-GFP and NEP but the translation of two distinct and separate proteins.

This procedure begins with the disruption of the splice acceptor site in the NEP ORF via site-directed mutagenesis of the RNA Polymerase I driven (pPol-I) NS rescue plasmid (Fodor et al., 1999; Neumann et al., 1999). This is followed by PCR amplification of three ORFs: the NS1 ORF including the 5' genomic non-coding region but lacking the 3' stop codon, the GFP ORF including the 3' 2A stop/start motif, and the NEP ORF including the 3' genomic non-coding region. The resulting PCR products will be incorporated by homologous recombination into the *SapI* linearized pDZ vector using Infusion Cloning (Clontech). Insertion via the *SapI* site ensures defined virus-specific 5' and 3' genomic ends, as *SapI* cuts beyond the restriction enzyme recognition site. Transformants are selected for Ampicillin resistance, and proper insertion of all three genes is confirmed by restriction enzyme digest and sequencing. The constructed pDZ NS plasmid encoding for the NS1-GFP fusion will then be ready for transfection-based virus rescues.

Materials

RNA Polymerase I driven (pPol-I) NS plasmid for IAV PR/8/34
RNA Polymerase I/Polymerase II bidirectional (pDZ) vector for IAV rescues
pMAXGFP vector (Amara)
DNase-RNase free water (Molecular Biology grade)
QuikChange Site-Directed Mutagenesis Kit (Agilent)
10mM dNTPs (dATP, dCTP, dGTP, dTTP) (see recipe)
PFU Ultra DNA Polymerase (Agilent)
Primers (see Table 1 for sequence):
 5'-SDM-NS-SAnull-F
 3'-SDM-NS-SAnull-R
 5'-INFU-NCR-NS1-F
 3'-INFU-NS1-noSTOP-R
 5'-INFU-GFP-F
 3'-INFU-GFP-2A-R
 5'-INFU-NEP_{ex1/2}-F
 3'-INFU-NEP-NCR-R
 5'-Sequencing
 3'-Sequencing
SapI restriction enzyme (New England Biolabs)
1kb Plus DNA Ladder (Fermentas)
6x DNA Loading Dye (Fermentas)
1x TAE (eg. Fisher)
1% Agarose-TAE gel (see recipe)
10mg/ml Ethidium Bromide (Fisher)
QIAquick gel extraction kit (Qiagen)
Infusion Cloning Kit (Clontech)
XbaI restriction enzyme (New England Biolabs)
LB agar plates supplemented with 100μg/ml Ampicillin (see recipe)
LB media supplemented with 100μg/ml Ampicillin (see recipe)
100mg/ml Ampicillin (see recipe)
QIAprep Spin Miniprep kit (Qiagen)
Thin-walled 0.2ml PCR tubes
Thermal Cycler
1.7ml microcentrifuge tubes

37°C incubator (rotating and non-rotating)

15ml polypropylene tubes (eg. BD-Falcon)

Horizontal gel apparatus and associated parts for agarose gels (eg. Bio-Rad)

Power supply (eg. BioRad)

UV transilluminator

Disrupt the splice acceptor site in NS by site-directed mutagenesis—Prior to reorganization of the NS genomic segment and insertion of the GFP gene, the splice acceptor site for NEP must be disrupted. As the bidirectional rescue (pDZ) plasmids are ill suited for site-directed mutagenesis due to high GC-content, the RNA Polymerase I (pPol-I) driven plasmid for the NS segment will be used for site-directed mutagenesis and subsequent cloning of the NS1 and NEP ORFs.

- 1 Mutate **TCCAGG** to **CCCGCC**, located at nucleotide position 524/527 in the NS segment, in the pPol-I NS plasmid using the QuikChange site-directed mutagenesis kit per the manufacturer's protocol. Use 50ng of the pPol-I NS plasmid as template; use 1μl each of 100μM 5'-SDM-NS-SAnull-F and 100μM 3'-SDM-NS-SAnull-R primers (Table 1) in the PCR reaction mix.

A primer design program is available online for the QuikChange Site-Directed Mutagenesis kit. In brief, each primer contains approximately 15 nucleotides of homology flanking the region to be mutated.

- 2 Select for mutated clones by plating the transformed bacteria onto LB agar supplemented with Ampicillin (100 μg/ml). Incubate plates upside down in a 37°C incubator overnight.
- 3 Pick 3–4 colonies and inoculate each into LB media supplemented with Ampicillin in 15ml polypropylene tubes. Grow overnight in a 37°C rotating incubator.
- 4 Isolate DNA from the above cultures using the QIAprep Spin Miniprep kit per the manufacturer's protocol.
- 5 Sequence clones with the primers listed in Table 1 to confirm the disruption of the splice acceptor site in the NS segment of IAV.

The resulting plasmid carries what is herein referred to as the NS 'Splice Acceptor null' (SA-null) gene.

PCR amplify and gel purify NS1 SA-null ORF

- 6 PCR amplify the NS1 SA-null ORF (with intact 5' non-coding region) from the pPol-I NS SA-null gene in thin walled 0.2ml PCR tubes in a thermocycler using the *PFU* Ultra DNA Polymerase enzyme, per the manufacturer's protocol. Use 50ng of the pPol-I NS SA-null plasmid as template; use 1μl each of 10μM 5'-INFU-NCR-NS1-F and 10μM 3'-INFU-NS1-noSTOP-R primers (Table 1), and 1μl of 10mM dNTPs in the PCR reaction mix. To generate the ~750 nt fragment with the above listed primers, change the cycling parameters as follows: 52°C for the annealing temperature, 1 minute for the extension time, and 30 cycles total.

A primer design program is available online for the Infusion Cloning kit. In brief, each primer contains approximately 15 nucleotides of homology flanking the gene of interest, in this case 15 nt of the pDZ

backbone on the 5' end and 15 nt of the GFP ORF on the 3' end. In addition, the reverse primer lacks the stop codon present for the NS1 ORF in order to generate the GFP fusion properly.

- 7 Pour a 1% agarose-TAE gel containing 1µl of 10mg/ml ethidium bromide per 100ml solution. Add 1/6 volume of 6x DNA loading dye to each PCR sample and load entire reaction onto gel. Also load the 1kb Plus DNA ladder for size verification. Run the gel in 1x TAE until the bands of the loading dye separate, approximately three quarters of the way down the gel.
- 8 Visualize the PCR product on a UV transilluminator (on the lowest setting possible). Cut the ~750 nt band using a clean razor blade, being sure to remove excess agarose gel. Place the gel slice in a clean 1.7ml microcentrifuge tube.

Do not expose the PCR product to UV light for extended periods of time. This may result in DNA nicking and poor quality.

- 9 Purify the PCR product from the agarose gel matrix using the QIAquick gel extraction kit, per the manufacturer's protocol.

Perform the optional Buffer QG Wash to ensure removal of any residual agarose.

- 10 Elute the NS1 SA-null ORF PCR product from the column with 50µl Molecular Biology grade DNase-RNase free water.

PCR amplify and gel purify GFP-2A ORF

- 11 PCR amplify the GFP ORF from the pMAXGFP plasmid in thin walled 0.2ml PCR tubes in a thermocycler using the *PFU* Ultra DNA Polymerase enzyme, per the manufacturer's protocol. Use 50ng of the pMAXGFP plasmid as template; use 1µl each of 10µM 5'-INFU-GFP-F and 3'-INFU-GFP-2A-R primers (Table 1), and 1µl of 10mM dNTPs in the PCR reaction mix. To generate the ~730 nt fragment with the above listed primers, change the cycling parameters as follows: 52°C for the annealing temperature, 1 minute for the extension time, and 30 cycles total.

These primers contain the necessary 15 nt of the NS1 ORF on the 5' end and 15 nt of the NEP ORF on the 3' end. In addition, the reverse primer includes a GSG linker and a portion of 2A ribosome stop/start sequence for insertion downstream of the NS1-GFP fusion ORF.

- 12 Repeat steps 7 through 10 to cut the ~730 nt band and obtain the purified GFP-2A ORF PCR product.

PCR amplify and gel purify NEP SA-null ORF

- 13 PCR amplify the NEP SA-null ORF (with intact 3' non-coding region) from the pPol-I NS SA-null gene in thin walled 0.2ml PCR tubes in a thermocycler using the *PFU* Ultra DNA Polymerase enzyme, per the manufacturer's protocol. Use 50ng of the pPol-I NS SA-null plasmid as template; use 1µl each of 10µM 5'-INFU-NEPex1/2-F and 10µM 3'-INFU-NEP-NCR-R primers (Table 1), and 1µl of 10mM dNTPs in the PCR reaction mix. To generate the ~435 nt fragment with the above listed primers, change the cycling parameters as follows: 52°C for the annealing temperature, 1 minute for the extension time, and 30 cycles total.

These primers contain the necessary ~15 nt of the GFP-2A ORF on the 5' end and 15 nt of the pDZ backbone on the 3' end. In addition, the forward primer contains the entire first exon and the first 21nt of the second exon of the NEP ORF.

- 14 Repeat steps 7 through 10 to cut the ~435nt band and obtain the purified NEP SA-null ORF PCR product.

Clone NS1 SA-null ORF, GFP-2A ORF, and NEP SA-null ORF into pDZ

- 15 Digest the pDZ vector with *SapI* at 37°C for 3–4 hours, per the manufacturer's protocol.

As the *SapI* restriction enzyme recognition sequences on the 5' and 3' ends of the multi-cloning site in the pDZ backbone are different, it is unnecessary to dephosphorylate the ends of the digested vector.

- 16 Repeat steps 7 through 10 to cut the ~4920 nt band and obtain the linearized pDZ backbone.
- 17 Perform Infusion Cloning using the three PCR products generated in steps 10, 12, and 14 and the linearized pDZ vector generated in step 16, per the manufacturer's protocol.
- 18 Select for recombinant clones by plating 1/5th of the transformed bacteria onto LB agar supplemented with Ampicillin. Incubate plates upside down in a 37°C incubator overnight.
- 19 Pick 6–12 colonies and inoculate each into LB media supplemented with Ampicillin in 15ml polypropylene tubes. Grow overnight in a 37°C rotating incubator.
- 20 Isolate DNA from the above cultures using the QIAprep Spin Miniprep kit per the manufacturer's protocol.
- 21 Screen for clones by digesting with *XbaI* at 37°C for 2hours, per the manufacturer's protocol. Visualize digest by gel electrophoresis as detailed in step 7.

Digestion with *XbaI* should result in three bands with sizes: 4500nt (pDZ backbone), 2100nt (NS-GFP segment with pDZ sequence) and 125nt (portion of pDZ vector).

- 22 Sequence clones with the 5'- and 3'-sequencing primers listed in Table 1 to confirm the proper insertion of NS1-GFP and NEP into the pDZ backbone.

The resulting plasmid is herein referred to as pDZ-NS-GFP.

BASIC PROTOCOL 2

Rescue of Infectious Recombinant NS1-GFP IAV

This protocol describes the rescue of infectious recombinant NS1-GFP IAV using reverse genetics and tissue culture based methodologies. As the RNA genome of IAV is of negative sense polarity, the rescue procedure must include a delivery vehicle for the negative sense genomic segments as well as expression of the viral proteins, which is achieved through use of the bidirectional-based pDZ rescue system (Figure 1).

The procedure begins with the transfection of a co-culture of Human Embryonic Kidney (HEK) 293 cells and Madin Darby Canine Kidney (MDCK) cells with the described rescue

plasmids, including the constructed pDZ-NS-GFP rescue plasmid from Basic Protocol 1, step 22. The HEK293 cells are highly transfectable, allowing for the delivery of all eight plasmids to a single cell for viral RNA, protein, and virus production. However, HEK293 cells do not efficiently grow IAV, thus the MDCK cells are used to amplify the few progeny virions produced by the HEK293 cells after transfection. Alternatively, an egg-based amplification can be performed with transfected HEK293 cells by injecting the HEK293 cell pellet into 8-day old eggs, as described by Szretter et al., 2006 (Szretter et al., 2006).

Following transfection of the HEK293/MDCK co-culture, progeny virions will be released into the supernatant. To prevent amplification of a potential quasi-species population post-rescue, supernatants are then subjected to plaque purification followed by amplification. As IAV causes cytopathic effect (CPE) in MDCK cells, a high dilution of virus can cause plaque formation, a focal point of clearing in the cell monolayer that represents a region of cells infected by and dying from a single virus particle. Plaque purification can then be performed, whereby a single plaque is used to inoculate a fresh pass of MDCK cells for viral amplification and stock generation.

The following protocol discusses the steps needed to rescue recombinant NS1-GFP IAV using reverse genetics and generate a virus stock from a single clone of NS1-GFP IAV. Through this process, infection and plaque formation can be traced by GFP expression, as cells infected by the virus will express GFP. Thus, infection can be monitored at all steps by fluorescence microscopy. Viral titers can either be determined by standard plaque assay for calculation of plaque forming units per milliliter, or by fluorescence for calculation of fluorescent focal units per milliliter.

Materials

Human Embryonic Kidney (HEK) 293 cells (ATCC CRL-1574)

Madin Darby Canine Kidney (MDCK) cells (ATCC CCL-34)

1× Dulbecco's Modified Eagle Medium (GIBCO)

Fetal Bovine Serum (FBS), heat inactivated (Hyclone, GIBCO)

100× Penicillin/Streptomycin Solution (Cellgro)

1× MDCK Growth Medium (see recipe)

RNA Polymerase I/Polymerase II bidirectional (pDZ) vectors for IAV PR8 strain rescues:

pDZ-PB2

pDZ-PB1

pDZ-PA

pDZ-HA

pDZ-NP

pDZ-NA

pDZ-M

pDZ-NS

pDZ-NS-GFP

Lipofectamine 2000 (Invitrogen)

Opti-MEM Reduced Serum Medium (GIBCO)

1mg/ml TPCK-trypsin (Trypsin from bovine pancreas; see recipe)

1× PBS, sterile (GIBCO)

35% Bovine Serum Albumin, sterile (MP Biomedical)

1× Infection Medium (see recipe)

2× Plaque Assay Medium (see recipe)

1.2% Agar (see recipe)

15-cm tissue culture treated dishes

6-well tissue culture treated dishes

Water-jacketed, 37°C, 5% CO₂ humidified incubator

15-ml polypropylene tubes (eg. BD-Falcon)

200 μ l pipet tip, sterile

2-ml cryovials

Transfect cells with IAV NS1-GFP rescue plasmids

- 1** Culture HEK293 cells in 15-cm tissue culture treated dishes to 80% confluency in 1× DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin at 37°C, 5% CO₂. Culture MDCK cells in 15-cm tissue culture treated dishes to 80% confluency in 1× MDCK Growth Medium at 37°C, 5% CO₂.

Do not let cells reach 100% confluency, as this will decrease transfection and rescue efficiency.

- 2** Transfect a mix of HEK293 cells and MDCK cells in Opti-MEM per the manufacturer's protocol for 6-well dishes, using 15-ml polypropylene tubes and the following per well:

1e⁶ HEK293 cells

5e⁵ MDCK cells

0.5 μ g pDZ-PB2

0.5 μ g pDZ-PB1

0.5 μ g pDZ-PA

0.5 μ g pDZ-HA

0.5 μ g pDZ-NP

0.5 μ g pDZ-NA

0.5 μ g pDZ-M

0.5 μ g pDZ-NS-GFP (from Basic Protocol 1, step 22)

8 μ l Lipofectamine 2000

In order to increase the chances of rescuing recombinant IAV, it is important to perform multiple transfections with the above plasmid mix (5–10 transfections). In addition, a positive control well can be included in this step by substituting the pDZ-NS-GFP in the above

mix for the wildtype pDZ-NS plasmid. CAUTION: Avoid cross-contamination between cells transfected with pDZ-NS-GFP or pDZ-NS; it is helpful to keep the positive control (pDZ-NS) transfection in a separate tissue culture dish.

Biosafety concerns: If working with a highly pathogenic strain of IAV, rescue transfections can be performed under Biosafety Level 2 conditions and transferred to the appropriate biocontainment level immediately.

- 3 Incubate transfected cells for 8 – 12 hours at 37°C, 5% CO₂.
- 4 Carefully aspirate the Opti-MEM/Lipofectamine mix from the transfected cells, and replace with 1.5ml of 1× Infection Medium + 1.5µl TPCK-trypsin (1mg/ml).
- 5 Incubate transfected cells for an additional 48 hours at 37°C, 5% CO₂. Check for cytopathic effect (CPE) and/or GFP expression in MDCK cells.
- 6 Store aliquots of supernatants from transfected cells demonstrating high CPE and/or high GFP expression at 4°C for subsequent plaque purification and amplification. Stocks are viable for up to one week. The presence of IAV can also be confirmed by hemagglutination assay (Szretter et al., 2006).

Plaque purify rescued NS1-GFP IAV from transfection supernatants

- 7 Seed MDCK cells (~1e6 cells/well) to complete confluency in 6-well tissue culture treated dishes.
Plan on needing one 6-well dish per supernatant sample.
- 8 Place 2× Plaquing Media (7ml per 6-well dish to be infected) in a 37°C water bath. Heat 1.2% Agar until dissolved and place in a 56°C water bath.
- 9 Dilute supernatant stocks in 1× PBS supplemented with 0.2% Bovine Serum Albumin and 1× Penicillin/Streptomycin. Perform five 1:10 dilutions for each supernatant sample demonstrating high CPE and/or high GFP expression.
- 10 Carefully aspirate the MDCK Growth Medium from the MDCK cells, and wash generously in 1× PBS.
It is important to wash the MDCK cells prior to infection; dead or unattached cells as well as the remaining FBS can inhibit viral infection.
- 11 Add the following inoculums to each 6-well dish of MDCK cells for infection:
 - Well #1: 200µl undiluted supernatant
 - Well #2: 200µl 1:10 dilution
 - Well #3: 200µl 1:100 dilution
 - Well #4: 200µl 1:1000 dilution
 - Well #5: 200µl 1:10,000 dilution
 - Well #6: 200µl 1:100,000 dilution
- 12 Incubate infected MDCK cells for 1 hour at 37°C, 5% CO₂, being sure to rock the dishes every ten minutes.
Cells left unattended will dry out, reducing cell viability.

- 13 Carefully aspirate the inoculums from the MDCK cells, and wash generously in 1× PBS.
It is important to wash the MDCK cells after infection to remove unattached virus.
- 14 Add TPCK-trypsin to the 2× Plaquing Media at a final concentration of 2μg/ml.
- 15 Mix 1:1 2× Plaquing Media + TPCK-trypsin (7ml) with 1.2% Agar (7ml) and immediately overlay cells, 2ml per well. Let the agar mix solidify at room temperature, approximately 10–15 minutes.
The agar overlay mix must not be too hot as to scald the cells, nor too cold as to solidify prior to overlaying. Test the temperature by touching the bottle with your gloved hand; it should not feel hot to the touch, but should remain warm enough to stay liquefied.
- 16 Incubate overlayed 6-well dishes upside down at 37°C, 5% CO₂ for 48–72 hours, until plaques are visible (opaque circular clearings in the cell monolayer).
If plaques are not visible after 72 hours, it is possible that there was not enough virus in the supernatant samples for plaquing. Use 200μl of the supernatant to inoculate a fresh pass of MDCK cells for amplification following steps 20–27. Once the supernatant from the initial rescue has been amplified once, proceed with the Basic protocol 2.
- 17 Outline and number the individual plaques with a marker on the underside of the 6-well dish. Confirm if the plaques are GFP positive by fluorescence microscopy. Do this for ~10 plaques for each positive supernatant sample.
- 18 Using a sterile 200μl pipet tip, stab the plaque through the agar overlay, and scratch the cells forming the plaque. Inoculate each plaque into 600μl of 1× PBS supplemented with 0.2% Bovine Serum Albumin and 1× Penicillin/Streptomycin.
- 19 Store inoculations at 4°C for subsequent rounds of amplification. Stocks are viable for 1 week.

Amplify infectious recombinant NS1-GFP IAV

- 20 Seed MDCK cells to complete confluency in 6-well tissue culture treated dishes.
Plan on needing one 6-well dish per supernatant sample.
- 21 Carefully aspirate the MDCK Growth Medium from the MDCK cells, and wash generously in 1× PBS.
It is important to wash the MDCK cells prior to infection; dead or unattached cells as well as the remaining FBS can inhibit viral infection.
- 22 Add 100μl inoculums to each 6-well dish of MDCK cells for infection:
- 23 Incubate infected MDCK cells for 1 hour at 37°C, 5% CO₂, being sure to rock the dishes every ten minutes.
Cells left unattended will dry out, reducing cell viability.
- 24 Carefully aspirate the inoculums from the MDCK cells, and wash generously in 1× PBS.
- 25 Add 2ml of 1X Infection Medium with 1μg/ml TPCK-Trypsin.

- 26 Incubate the infected MDCK cells for an additional 48 hours at 37°C, 5% CO₂. Check for CPE and/or GFP expression in MDCK cells.
- 27 Collect the supernatant from wells demonstrating ~30–40% CPE/GFP expression.
- 28 Aliquot supernatants into 2-ml cryovials and store at –80°C for subsequent virus titration as described by Szretter et al., 2006.

REAGENTS AND SOLUTIONS

10mM dNTPs (dATP, dCTP, dGTP, dTTP)

Per 1ml:

100µl of 100mM dATP (10mM final)

100µl of 100mM dCTP (10mM final)

100µl of 100mM dGTP (10mM final)

100µl of 100mM dTTP (10mM final)

600µl sterile DNase/RNase free H₂O

Mix, aliquot, and store at –20°C

1% Agarose-TAE gel

Per 100ml:

1g Agarose, Molecular Biology Grade (Eg. Fisher)

100ml 1× TAE (Eg. Fisher)

Microwave until completely dissolved

100mg/ml Ampicillin (sterile)

Per 10ml:

1g Ampicillin Sodium Salt (Eg. Fisher)

10ml Autoclaved ddH₂O

Filter sterilize with a 0.2-µm membrane

Aliquot and store at –20°C

LB agar plates supplemented with 100µg/ml Ampicillin

Per 1L:

10g Bacto-Tryptone (Eg. Fisher)

10g NaCl (Eg. Sigma)

5g Yeast Extract (Eg. Fisher)

15g Bacto agar (Eg. Fisher)

1L ddH₂O

Autoclave and let cool to 55°C

1ml of 100mg/ml Ampicillin (100µg/ml final)

Mix and quickly pour plates

Let solidify, package, and store upside down at 4°C

LB media supplemented with 100µg/ml Ampicillin

Per 1L:

10g Bacto-Tryptone (Eg. Fisher)

10g NaCl (Eg. Sigma)

5g Yeast Extract (Eg. Fisher)

1L ddH₂O

Autoclave and let cool completely

1ml of 100mg/ml Ampicillin (100µg/ml final)

Store at room temperature

1× MDCK Growth Medium

Per 500ml:

50ml 10× Eagle's Minimal Essential Medium (EMEM; Fisher; 1× final)

50ml Fetal Bovine Serum (FBS), heat inactivated (Hyclone; 10% final)

5ml 100× Penicillin/Streptomycin (Cellgro; 1× final)

5ml 100× (200mM) L-Glutamine (GIBCO; 1× (2mM) final)

10ml 7.5% NaHCO₃ (Sodium bicarbonate), sterile (Sigma; 0.15% final)

5ml 1M HEPES Buffer (Cellgro; 10mM final)

370ml Autoclaved ddH₂O

Filter sterilize with a 0.2-µm membrane

Store at 4°C

1mg/ml TPCK-trypsin (Trypsin from bovine pancreas)

Per 10ml:

10mg L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma)

10ml Autoclaved ddH₂O

Filter sterilize with a 0.2-µm membrane

Aliquot and store at -20°C

1× Infection Medium

Per 500ml:

50ml 10× Eagle's Minimal Essential Medium (EMEM; Fisher; 1× final)

3ml 35% Bovine Serum Albumin (BSA), sterile (MP Biomedical; 0.21% final)

5ml 100× Penicillin/Streptomycin (Cellgro; 1× final)

5ml 100× (200mM) L-Glutamine (GIBCO; 1× (2mM) final)

10ml 7.5% NaHCO₃ (Sodium bicarbonate), sterile (Sigma; 0.15% final)
5ml 1M HEPES Buffer (Cellgro; 10mM final)
417ml Autoclaved ddH₂O
Filter sterilize with a 0.2-μm membrane
Store at 4°C

2x Plaque Assay Medium

Per 500ml:
100ml 10× Eagle's Minimal Essential Medium (EMEM; Fisher; 2× final)
6ml 35% Bovine Serum Albumin (BSA), sterile (MP Biomedical; 0.42% final)
10ml 100× Penicillin/Streptomycin (Cellgro; 2× final)
10ml 100× (200mM) L-Glutamine (GIBCO; 1× (4mM) final)
30ml 7.5% NaHCO₃ (Sodium bicarbonate), sterile (Sigma; 0.45% final)
10ml 1M HEPES Buffer (Cellgro; 20mM final)
5ml 1% Diethylaminoethyl (DEAE)-Dextran Chloride (see recipe; final 0.01%)
329ml Autoclaved ddH₂O
Filter sterilize with a 0.2-μm membrane
Store at 4°C

1% Diethylaminoethyl (DEAE)-Dextran Chloride

Per 500ml:
5g Diethylaminoethyl (DEAE)-Dextran Chloride (MP Biomedical)
500ml Autoclaved ddH₂O
Microwave until completely dissolved
Filter sterilize with a 0.2-μm membrane
Store at 4°C

1.2% Agar

Per 200ml:
2.4g Agar (Oxoid)
200ml Autoclaved ddH₂O
Microwave until completely dissolved

COMMENTARY

Background Information

Influenza A virus (IAV) is a negative sense, segmented RNA virus of the *Orthomyxoviridae* family; infection results in an upper respiratory disease in humans ranging from subclinical symptoms to pulmonary pneumonia. IAV is the major cause of seasonal influenza epidemics and is solely responsible for sporadic influenza pandemics (Cox NJ et al., 2005; Palese P and Shaw ML, 2007). Nearly 226,000 hospitalizations and 36,000 deaths in the US alone can be

attributed annually to seasonal influenza infections (Fiore et al., 2008). Wild waterfowl are the natural reservoir of IAV; however, the virus circulates in numerous species including humans, birds, and swine (Neumann and Kawaoka, 2006). There are multiple serological subtypes of IAV, several of which can cause severe disease in humans as well as zoonotic infections across species (Webster et al., 1992).

IAV remains a constant threat to public health due to its ability to change its antigenicity (Cox NJ et al., 2005; Mathews et al., 2009; Palese P and Shaw ML, 2007). The IAV genome accumulates mutations during human circulation, which can change the antigenicity of the surface glycoproteins, hemagglutinin and neuraminidase. This process is known as antigenic drift, and results in loss of immune protection and susceptibility to re-infection. As such the current vaccine must be reformulated yearly to account for the newly circulating strains. Due to the segmented nature of the genome, IAV is also able to exchange genomic segments between distinct strains, resulting in the appearance of a completely new and foreign virus. This process is known as antigenic shift, which can lead to reassortment of the segments encoding for the surface glycoproteins, and potentially create a pandemic strain.

Although IAV has the ability to perpetually evade immunity, it is composed of only eight RNA segments. Through the use of splicing and alternative open-reading frames, the virus is able to produce a dozen proteins from the eight genomic segments. The pDZ rescue plasmids used in this unit were designed to have bidirectional promoters, in that they produce both negative sense viral RNA and positive sense mRNA for each genomic segment (Hoffmann et al., 2000; Quinlivan et al., 2005). By encoding for a single segment on each plasmid, transfection of all eight plasmids representing the eight segments of IAV into cells results in the production of infectious virus. This reverse genetics system allows us to accomplish many tasks, including but not limited to: easy generation of recombinant strains, construction of genetic reassortant viruses, creation of deletions or mutations of viral proteins, and insertion of fluorescent reporter genes into the IAV genome. The development of a fluorescent reporter IAV provides us with the unique ability to track infection, quantify replication, and identify infected cell populations *in vivo*. In addition, this reporter virus can be used to screen novel antiviral therapeutics.

Critical Parameters and Troubleshooting

Site-directed mutagenesis—Proper primer design is essential to the efficacy of the site-directed mutagenesis protocol, regardless of the manufacturer. Most manufacturers will provide an online tool for designing primers specifically for the experimental template. For the QuikChange kit (Agilent), the primers should have a minimum of 15 nucleotides of homology flanking the splice acceptor site that is to be disrupted. Efficiency of mutagenesis decreases with the more nucleotides that are changed per reaction; it is recommended to keep the changes to three nucleotides or less. If more nucleotide changes are needed, multiple rounds of mutagenesis can be employed. A powerful, high-fidelity DNA polymerase is included in this kit, as the PCR reaction generates the entire pPol-I NS SA-null plasmid from the supplied primers. Long extension times are crucial for complete template production, and the cycle numbers should be kept low as to prevent mutation by the DNA polymerase.

As the site-directed mutagenesis protocol relies on the use of a methylation-dependent restriction enzyme (for the QuikChange kit it is *DpnI*) to destroy the parent pPol-I NS plasmid, it is essential to use a pPol-I NS plasmid stock derived from a methylation competent strain of bacteria, such as DH5alpha. Otherwise, contaminating original plasmid will be present after site-directed mutagenesis. To ensure proper digestion of the original plasmid in each PCR reaction, a negative control should be included in the site-directed mutagenesis experimental set-up. This negative control should have the same amount of

original pPol-I NS plasmid used in the other reaction(s), but should not contain primers. In this way, only the unmutated pPol-I NS plasmid is present during digestion, and therefore a complete digestion will result in no bacterial colonies after transformation. Similar numbers in bacterial colonies for the negative control and primer-containing reaction(s) would signify an incomplete or problematic reaction.

Infusion cloning—Similarly to the site-directed mutagenesis protocol, Infusion cloning (Clontech) relies on the use of appropriate primers during the PCR amplification step. A primer design tool can be found on the manufacturer's website. As Infusion cloning utilizes homologous recombination to join the NS1, GFP, and NEP PCR products with the pDZ vector, ~15 nucleotides of homology are needed flanking each gene insertion. For the 5' and 3' ends, ~15 nucleotides of the vector should be included in the primers; for multiple PCR products, ~15 nucleotides of the adjacent PCR product should be included in the primers. Standard PCR considerations should be made for this step, such as: adequate extension time to ensure complete synthesis of the gene(s); use of a high fidelity DNA Polymerase (like PFU Ultra (Agilent)) to prevent accumulation of mutations in the DNA; and testing of various annealing temperatures to identify the specific conditions required for the designed primers. PCR amplification from a plasmid template should result in a single bright band for each product; if not, optimize the PCR reaction to increase yield.

Proper linearization of the vector is also essential to the Infusion cloning process. Allow sufficient time for complete linearization of the vector, in this case 3–4 hours for *SapI*, and be sure to only excise linear vector from the agarose gel. Use a clean razor blade for the different bands to be purified, so as not to cross-contaminate the DNA. As the two *SapI* restriction sites in the pDZ backbone result in ends that are not complementary, and as the Infusion cloning process does not rely on a DNA ligase, it is not necessary to dephosphorylate the ends of the digested vector. However, if this becomes a concern due to inadequate *SapI* digestion of pDZ vector, CIP (New England Biolabs) can be used to prevent self-ligation. Furthermore, include a negative control in the Infusion cloning reaction to ensure proper digestion of the vector – set up a reaction with vector but no insert(s), such that transformation will result in no bacterial colonies for a properly digested vector. Similar numbers in bacterial colonies for the negative control and insert-containing reaction(s) would signify a poor vector/insert preparation or problematic reaction.

Cell culture—Efficient transfection and subsequent viral rescues rely on healthy, contamination-free cells. Care should be taken to ensure that the HEK293 cells remain highly transfectable. Low passage numbers and passaging prior to complete confluency are examples of measures that can be taken to ensure the cells will have high transfection efficiencies. Furthermore, including a fluorescent control reaction can allow for direct monitoring of transfection efficiency; greater than 90% transfection should be achieved with the HEK293s used for viral rescues. Increasing the number of transfections performed with the rescue plasmids can increase the likelihood of rescuing recombinant IAV, as can use of clean, pure DNA rescue plasmids.

Likewise, healthy, clean MDCK cells are essential to the viral rescue process. Use of over-passed or over-confluent MDCK cells will greatly reduce the efficacy of IAV rescue. For plaque purification and viral titering, it should be confirmed beforehand that the cells are competent for plaque production. Test cells by performing plaque assays with a wildtype IAV stock of known titer. If no plaques are visible after two days, thaw and recover a new pass of MDCK cells.

Plasmid and virus verification—Rescue DNA plasmids and virus stocks should be confirmed by sequencing to prevent use of mutated IAV strains and maintain consistency

between stocks. Recombinant rescue plasmids should be confirmed prior to rescue; sequence the entire gene and flanking vector to ensure proper orientation and complete insertion. If aberrant point mutations are identified, site-directed mutagenesis can be used to revert these positions to wildtype sequence. Alternatively, the PCR amplification step can be performed again with a higher fidelity DNA polymerase. After successful rescue of recombinant IAV, use reverse transcription and PCR amplification to obtain enough DNA representing the NS-GFP gene for standard sequencing.

Anticipated Results

Site-directed mutagenesis and Infusion cloning are streamlined, efficient procedures. As only two nucleotides need to be changed, disruption of the splice acceptor site by site-directed mutagenesis should result in many positive clones. For the PCR amplifications, the pPol-I plasmid serves as the template, increasing the efficiency of accurate priming, and resulting in the production of bright, easily visualized single bands. The use of a bright single band for the inserts and vector will result in several positive clones for screening. After construction of the recombinant NS-GFP rescue plasmid, sequencing should confirm disruption of the splice acceptor site, as well as proper positioning of the NS1, GFP, and NEP genes relative to the pDZ backbone. Rescue transfections with clean, confirmed DNA plasmids will result in NS1-GFP IAV rescue; multiple transfections and MDCK co-cultures will increase the likelihood of rescue. The resulting virus will encode for GFP, which can be used to trace infection and identify infected cells via fluorescence microscopy.

Time Considerations

The overall procedure to construct and rescue recombinant GFP IAV can take approximately 3 – 5 weeks. Site-directed mutagenesis can be completed in 3 – 4 days after primer synthesis and delivery. PCR amplification and Infusion cloning should take roughly the same number of days, although PCR optimization may require additional time. Construction of the rescue plasmid in all should take 1 – 2 weeks. Rescue plasmid transfection and MDCK co-culture will take 3 days; however, if an amplification step is required prior to plaque purification, this will result in an additional 2 – 3 day delay. Plaque purification, amplification, and titering can take upwards of one week. Anticipate approximately 2 – 3 weeks for complete rescue and stock generation.

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Figure 1. Schematic representation of bidirectional IAV rescue system

In this bidirectional system, mRNA is transcribed from a beta-actin promoter by RNA polymerase II and viral genomic RNA is transcribed by RNA polymerase I.

Figure 2. Engineering of GFP reporter gene in IAV genome

(A) Comparison of the NS segment of wild-type and GFP reporter virus. (Top) The wild-type NS segment expresses NS1/NEP proteins by mRNA splicing. (Bottom) The GFP virus expresses both NS1-GFP and NEP proteins from a single mRNA via a ribosome start/stop at the porcine teschovirus-1 2A (PTV-1 2A) site. SD/SA - Splice donor/acceptor sites. (B) Sequence of splice acceptor site before and after site-directed mutagenesis. (C) Sequence information on the junction regions of GFP, PTV-1 2A site, and NEP.

Table 1

Primers used for generating NS1-GFP influenza virus.

5'-SDM-NS-SAnull-F	CACCATTCCTTCTCTCCCGGGACATACTGCTGAGG
3'-SDM-NS-SAnull-R	CCTCAGCAGTATGTCCCGGGAGAGAAGGCAATGGTG
5'-INFU-NCR-NS1-F	CGACCTCCGAAGTTGGGGGGGAGCAAAAGCAGG
3'-INFU-NS1-noSTOP-R	GGCGGGCATGCCGGACCAACTTCGCTTCTAATTGTTCC
5'-INFU-GFP-F	GGGTCCGGCATGCCCGCCATGAAGATCGAGTGCC
3'-INFU-GFP-2A-R	TTCTTCCACATCGCCCGCCTGTTTCAGCAGGCTAAAGTGGTCGCGCCGCTGCCGGCGAATGCGATCGGGGTCTT
5'-INFU-NEPex1/2-F	GGCGATGTGGAAGAAAACCCGGGCCCGATGGATCCAAACACTGTGTCAAGCTTTCAGGACATACTGCTGAGGATGTC
3'-INFU-NEP-NCR-R	GCATTTTGGGCCGCGGGTTATTAGTAGAAACAAGG
5' sequencing	CGGTACCCGGGGATCCTCTAG
3' sequencing	GCGACCTCCCGGCCCGGGG