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Simplified Reverse Genetics Method to Recover Recombinant Rotaviruses Expressing Reporter Proteins

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Cover letter

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Re: JoVE61039

Please consider our revised manuscript entitled "*Simplified reverse genetics method to recover recombinant rotaviruses expressing reporter proteins*" for publication in ***Jove***. In leu of the reviewers' suggestions, the above is a modified title. Previously it was *"Recovery of recombinant rotavirus expressing fluorescent reporter protein"*.

In addition to the text for the manuscript, we have uploaded three figure files (.psd, 300 dpi), Table of Materials (.xlsx), and specific responses to editorial and reviewers' comments..

I hope the manuscript is now suitable for production.

Sincerely,

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TITLE:

Simplified Reverse Genetics Method to Recover Recombinant Rotaviruses Expressing Reporter Proteins

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KEYWORDS:

rotavirus, reverse genetics, fluorescent protein, recombinant virus, recombinant rotaviruses, viral expression vector

SUMMARY:

Generation of recombinant rotaviruses from plasmid DNA provides an essential tool for the study of rotavirus replication and pathogenesis, and the development of rotavirus expression vectors and vaccines. Herein, we describe a simplified reverse genetics approach for generating recombinant rotaviruses, including strains expressing fluorescent reporter proteins.

ABSTRACT:

Rotaviruses are a large and evolving population of segmented double-stranded RNA viruses that cause severe gastroenteritis in the young of many mammalian and avian host species, including humans. With the recent advent of rotavirus reverse genetics systems, it has become possible to use directed mutagenesis to explore rotavirus biology, modify and optimize existing rotavirus vaccines, and develop rotavirus multitarget vaccine vectors. In this report, we describe a simplified reverse genetics system that allows the efficient and reliable recovery of recombinant rotaviruses. The system is based on co-transfection of T7 transcription vectors expressing full-length rotavirus (+)RNAs and a CMV vector encoding an RNA capping enzyme into BHK cells constitutively producing T7 RNA polymerase (BHK-T7). Recombinant rotaviruses are amplified by overseeding the transfected BHK-T7 cells with MA104 cells, a monkey kidney cell line that is highly permissive for virus growth. In this report, we also describe an approach for generating recombinant rotaviruses that express a separate fluorescent reporter protein through the introduction of a 2A translational stop-restart element into genome segment 7 (NSP3). This approach avoids deleting or modifying any of the viral open reading frames, thus allowing the production of recombinant rotaviruses that retain fully functional viral proteins while expressing a fluorescent protein.

INTRODUCTION:

Rotaviruses are major causes of severe gastroenteritis in infants and young children, as well as the young of many other mammalian and avian species[1]. As members of the *Reoviridae* family, rotaviruses have a segmented double-stranded RNA (dsRNA) genome. The genome segments are contained within a nonenveloped icosahedral virion formed from three concentric layers of protein[2]. Based on sequencing and phylogenetic analysis of the genome segments, nine species of rotavirus (A–D, F–J) have been defined[3]. Those strains comprising rotavirus species A are responsible for the vast majority of human disease[4]. The introduction of rotavirus vaccines into childhood immunization programs beginning in the last decade is correlated with significant reductions in rotavirus mortality and morbidity. Most notably, the number of rotavirus-associated childhood deaths has decreased from approximately 528,000 in 2000 to 128,500 in 2016[4,5]. Rotavirus vaccines are formulated from live attenuated strains of the virus, with 2 to 3 doses administered to children by 6 months of age. The large number of genetically diverse rotavirus strains circulating in humans and other mammalians species, combined with their ability to rapidly evolve through mutagenesis and reassortment, may lead to antigenic changes in the types of rotaviruses infecting children[6,7,8]. Such changes may undermine the efficacy of existing vaccines, requiring their replacement or modification.

The development of fully plasmid-based reverse genetics systems enabling manipulation of any of the 11 rotavirus genome segments was only recently achieved[9]. With the availability of these systems, it has become possible to unravel molecular details of rotavirus replication and pathogenesis, to develop improved high-throughput screening methods for anti-rotavirus compounds, and to create new potentially more effective classes of rotavirus vaccines. During rotavirus replication, capped viral (+)RNAs not only guide the synthesis of viral proteins, but also serve as templates for the synthesis of progeny dsRNA genome segments[10,11]. All rotavirus reverse genetics systems described to date rely on the transfection of T7 transcription vectors into mammalian cell lines as a source of cDNA-derived (+)RNAs used in recovering recombinant viruses[9,12,13]. Within the transcription vectors, full-length viral cDNAs are positioned between an upstream T7 promoter and downstream hepatitis delta virus (HDV) ribozyme such that viral (+)RNAs are synthesized by T7 RNA polymerase that contain authentic 5’ and 3’-termini (**Figure 1A**). In the first-generation reverse genetics system, recombinant viruses were made by transfecting baby hamster kidney cells expressing T7 RNA polymerase (BHK-T7) with 11 T7 (pT7) transcription vectors, each directing synthesis of a unique (+)RNA of the simian SA11 virus strain, and three CMV promoter-drive expression plasmids, one encoding the avian reovirus p10FAST fusion protein and two encoding subunits of the vaccinia virus D1R-D12L capping enzyme complex[9]. Recombinant SA11 viruses generated in transfected BHK-T7 cells were amplified by overseeding with MA104 cells, a cell line permissive for rotavirus growth. A modified version of the first-generation reverse genetics system has been described that no longer uses support plasmids[12]. Instead, the modified system successfully generates recombinant rotaviruses simply by transfecting BHK-T7 cells with the 11 SA11 T7 transcription vectors, with the caveat that vectors for the viral factory (viroplasm) building blocks (nonstructural proteins NSP2 and NSP5) are added at levels 3-fold higher than the other vectors[14,15]. Modified versions of the reverse genetics system have also been developed that support the recovery of the human KU and Odelia

strains of rotavirus[16,17]. The rotavirus genome is remarkably amenable to manipulation by reverse genetics, with recombinant viruses generated to date with mutations introduced into VP4[18], NSP1[9], NSP2[19], NSP3[20,21], and NSP5[22,23]. Among the most useful viruses generated so far are those that have been engineered to express fluorescent reporter proteins (FPs)[9,12,21,24,25].

In this publication, we provide the protocol for the reverse genetics system that we use in our laboratory to generate recombinant strains of SA11 rotavirus. The key feature of our protocol is co-transfection of BHK-T7 cells with the 11 pT7 transcription vectors (modified to include 3x levels of the pT7/NSP2SA11 and pT7/NSP5SA11 vectors) and a CMV expression vector encoding the African swine fever virus (ASFV) NP868R capping enzyme[21] (**Figure 2**). In our hands, presence of the NP868R plasmid leads to the production of higher titers of recombinant viruses by transfected BHK-T7 cells. In this publication, we also provide a protocol for modifying the pT7/NSP3SA11 plasmid such that recombinant viruses can be generated that express not only the segment 7 protein product NSP3 but also a separate FP. This is accomplished by re-engineering the NSP3 open reading frame (ORF) in the pT7/NSP3SA11 plasmid to contain a downstream 2A translational stop-restart element followed by an FP ORF (**Figure 1B**)[24,26]. Through this approach, we have generated recombinant rotaviruses expressing various FPs: UnaG (green), mKate (far-red), mRuby (red), TagBFP (blue), CFP (cyan), and YFP (yellow)[24,27,28]. These FP-expressing rotaviruses are made without deleting the NSP3 ORF, thus yielding viruses that are expected to encode a full complement of functioning viral proteins.

PROTOCOL:

1. Media preparation and cell culture maintenance

1.1. Obtain baby hamster kidney cells constitutively expressing T7 RNA polymerase (BHK-T7) and African green monkey kidney MA104 cells.

NOTE: BHK-T7 (or BSR-T7) cells are not commercially available, but are a common cell line of laboratories using reverse genetics to study RNA virus biology. The BHK-T7 cell line used in this protocol was obtained from Dr. Ursula J. Buchholz (National Institutes of Health, Bethesda, MD, USA), a co-developer of the original BHK cell line expressing T7 RNA polymerase[29]. MA104 cells are available from European Collection of Authenticated Cell Cultures (ECACC) and from American Type Culture Collection (ATCC).

1.2. Prepare the following culture media in a sterile environment, preferably a Class II biological safety cabinet. Store media in the dark at 4 °C and warm to 37 °C immediately prior to use.

NOTE: Sources of media components are provided in **Table of Materials**.

1.2.1. To prepare DMEM incomplete medium, combine 500 mL of Dulbecco's modified Eagle's minimum essential medium (MEM) containing 4.5 g/L glucose and 1% glutamine with 5 mL of 100x pen-strep.

1.2.2. To prepare DMEM complete medium, combine 500 mL of DMEM incomplete medium with 25 mL of fetal bovine serum (FBS).

1.2.3. To prepare GMEM incomplete medium, combine 500 mL of Glasgow minimum essential medium, 5 mL of 100x glutamine, 50 mL of tryptose-phosphate broth (TPB), 5 mL of 100x nonessential amino acids (NEAA), and 5 mL of 100x pen-strep.

1.2.4. To prepare GMEM complete medium, combine 500 mL of GMEM incomplete medium with 25 mL of heat-inactivated FBS.

1.2.5. To prepare GMEM+G complete medium, add 10 mL of 50 mg/mL geneticin (G418) to 500 mL of GMEM complete medium.

1.2.6. To prepare SMEM incomplete medium, combine 500 mL of Joklik's modified Eagle's MEM, 5 mL of 100x glutamine, 50 mL of TPB, 5 mL of 100x NEAA, and 5 mL of 100x pen-strep.

1.3. Culture MA104 cells in T75 or T175 flasks containing 12 or 25 mL of DMEM complete medium, respectively. To passage MA104 cells that have reached 100% confluency, rinse the cell monolayer 2x with phosphate-buffered saline (PBS), dissociate with trypsin (0.05%)-EDTA (0.1%) solution, and resuspend in 5 (T75 flask) or 10 mL of (T175 flask) of DMEM incomplete medium.

1.3.1. Place 0.5−1.0 mL of resuspended cells in fresh flasks and bring to the appropriate final volume by adding DMEM complete medium. Place flasks in a 37 °C, 5% CO_2 incubator.

1.4. Propagate BHK-T7 cells in T75 flasks, alternating between the use of GMEM and GMEM+G complete medium with each round of passage. To subculture BHK-T7 cells that have reached 100% confluency, rinse the cell monolayer 2x with PBS, dissociate with trypsin-EDTA solution, and resuspend in 5 mL of medium.

1.4.1. After placing 15 mL of GEM or GMEM+G complete medium in a fresh T75 flask, add four drops of resuspended BHK-T7 cells. Place flask in a 37 °C, 5% CO_2 incubator.

2. Plasmid preparation

2.1. Obtain the following plasmids expressing rotavirus SA11 (+)RNAs from Addgene: pT7/VP1SA11, pT7/VP2SA11, pT7/VP3SA11, pT7/VP4SA11, pT7/VP6SA11, pT7/VP7SA11, pT7/NSP1SA11, pT7/NSP2SA11, pT7/NSP3SA11, pT7/NSP4SA11, and pT7/NSP5SA11. Obtain the plasmid expressing the ASFV capping enzyme (pCMV/NP868R) from the authors[21].

NOTE: Modified pT7/NSP3SA11 plasmids engineered to express a FP through the activity of a 2A translational element (pT7/NSP3-2A-3xFL-FP) can also be obtained from the authors[24,26]. pT7/NSP3-2A-3xFL-FP plasmids expressing the following FPs are available: UnaG (green), mKate (far-red), mRuby (red), TagBFP (blue), CFP (cyan), and YFP (yellow)[24].

2.2. Transform plasmids into competent *E. coli* DH5α and spread bacteria onto Luria broth agar plates containing the appropriate antibiotic. Grow bacterial cultures picked from individual colonies and prepare smaller amounts of plasmid (20 µg) using a spin miniprep purification kit (**Table of Materials**), according to the manufacturer's instructions. Prepare larger amounts of plasmid from bacterial cultures using midi and maxi purification kits (**Table of Materials**).

NOTE: Plasmids suitable for use in the reverse genetics system have also been prepared with other plasmid purification kits. It is not necessary to prepare plasmids using kits specifically designed to generate endotoxin-free material.

2.3. Adjust concentrations of purified plasmids to 1 mg/mL in nuclease-free molecular biology grade water. Check plasmid purity by confirming a 260/280 absorbance ratio of ~1.8 using a spectrophotometer. Also, use electrophoresis on 0.8% agarose gels to verify that plasmids are predominantly supercoiled.

2.4. Aliquot purified plasmids into 0.5 mL sterile microcentrifuge tubes, each containing 10 µL. Store plasmids at -80 °C.

3. Generation of recombinant virus

NOTE: Human and animal rotavirus research, including the generation and characterization of recombinant rotavirus strains, must be handled under Biosafety Level 2 (BSL-2) conditions and will require prior approval by the Institutional Biosafety Committee (IBC). Appropriate BSL-2 laboratory conditions are described in *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) produced by the Centers for Disease Control and Prevention (CDC)[30].

3.1. Day 1: seeding of BHK-T7 cells into 12-well plates

3.1.1. Rinse a freshly confluent monolayer of BHK-T7 cells contained in a T75 flask 2x with PBS. Disrupt the cell monolayer with trypsin-EDTA solution and resuspend the cells in 5 mL of GMEM complete medium.

3.1.2. Use an automated cell counter and trypan-blue solution to determine the concentration of viable BHK-T7 cells in the medium. Seed cells into 12-well cell culture plates, with each well containing 2 x 10^5 cells in a total volume of 1 mL of GMEM (G418-free) complete medium. Incubate in a 37 °C, 5% CO_2 incubator. The cells should reach 80−90% confluency by day 2.

3.2. Day 2: transfection of BHK-T7 cells with plasmid mixtures

3.2.1. To prepare the plasmid mixture, combine the following in a 0.5 mL microcentrifuge tube using plasmid stocks adjusted to 1 mg/mL: 0.8 µL each of SA11 pT7 plasmids VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP3 and NSP4, 2.4 µL each of SA11 pT7 plasmids NSP2 and NSP5, and 0.8 µL of pCMV/NP868R. Gently mix plasmids by tapping the tube and collect contents by pulse

centrifugation. To prepare recombinant viruses expressing FPs, replace pT7/NSP3SA11 with the appropriate pT7/NSP3-2A-3xFL-FP plasmid.

NOTE: Plasmid mixtures should be stored on ice until used. A separate tube should be prepared for each transfection.

3.2.2. To prepare the plasmid/reduced serum medium/transfection reagent mixture: Add 110 µL of prewarmed (37 °C) reduced serum medium (**Table of Materials**) to each plasmid mixture and mix by gently pipetting up and down. Afterwards, add 32 µL of transfection reagent (**Table of Materials**) to each plasmid/reduced serum medium mixture; this yields a concentration of 2.5 µL of transfection reagent per µg of plasmid in mixtures. Vortex mixtures gently and incubate at room temperature for 20 min.

3.2.3. During the 20 min incubation period, rinse BHK-T7 cells in 12-well plates (prepared on day 1) once with 2 mL of GMEM incomplete media. Afterwards, add 1 mL of SMEM incomplete medium to each well and return plates to the incubator.

3.2.4. After the 20 min incubation period, add the plasmid/reduced serum medium/transfection mixture drop-by-drop to each well of the 12-well plates using a 200 µL pipettor. Gently rock plates and return to a 37 °C, 5% CO_2 incubator.

3.3. Day 4: overseeding transfected BHK-T7 cells with MA104 cells

3.3.1. Rinse a freshly confluent monolayer of MA104 cells contained in a T75 flask 2x with PBS. Disrupt the monolayer using trypsin-EDTA solution and resuspend cells in 5 mL of DMEM complete medium.

3.3.2. Using an automated cell counter and trypan-blue solution, determine the concentration of viable MA104 cells in the medium. Adjust the concentration to 8 x 10^5 MA104 cells/mL in DMEM incomplete medium and add 0.25 mL of suspended cells (2 x 10^5 cells) dropwise to the wells containing transfected BHK-T7 cells.

3.3.3. Adjust the concentration of trypsin (porcine pancreatic type IX) in the medium to ~0.5 µg/mL by adding 0.8 µL of 0.1 mg/mL trypsin stock to each well.

NOTE: Trypsin stocks should be prepared in PBS, aliquoted, and stored at -20 °C.

3.3.4. Use the remaining MA104 cells to prepare 6-well plates that will be needed on day 7 to amplify recombinant viruses. To seed 6-well plates, dilute resuspended MA104 cells to a concentration of 1.5 x 10^5 cells/mL in DMEM complete medium and place 2 mL in each well. Place plates in a 37 °C, 5% CO_2 incubator.

3.4. Day 7: recovery and amplification of recombinant virus from transfected cells

3.4.1. Subject BHK-T7/MA104 cells in 12-well plates to three cycles of freeze-thaw under sterile conditions, moving plates between -20 °C freezer and a room temperature surface. After transferring lysates to 1.5 mL tubes, centrifuge tubes for 10 min at 500 x *g* (4 °C) to pellet large cellular debris. Collect supernatant and store at 4 °C (short term) or -20 °C (long term).

3.4.2. Wash MA104 monolayers in 6-well plates (prepared on day 4) 2x with PBS. Place 2 mL of DMEM incomplete medium to each well that contains 0.5 µg/mL trypsin. Add 300 µL of supernatant recovered from BHK-T7/MA104 cell lysates into wells and place plates in a 37 °C, 5% CO_2 incubator.

3.4.3. Incubate plates for 7 days or until complete cytopathic effects (CPE) are observed. Lyse MA104 cells in 6-well plates by three cycles of freeze-thaw, then transfer lysates to 1.5 mL microcentrifuge tubes. Pellet large cellular debris by centrifugation for 10 min at 500 x *g* (4 °C). Transfer the clarified cell lysates into 1.5 mL microcentrifuge tubes and store at -20 °C.

4. Plaque isolation of recombinant viruses

4.1. Activate viruses in 100 µL of clarified cell lysates by adding trypsin to a final concentration of 10 µg/mL and incubating at 37 °C for 1 h. Prepare a 10-fold serial dilution series ranging from 10^{-1} to 10^{-7} (1 mL each) in DMEM incomplete medium.

4.2. Rinse MA104 monolayers in 6-well plates 2x with 2 mL of PBS and once with DMEM incomplete medium. Add 400 mL of lysate dilutions in duplicate to the plates. Incubate the plates for 1 h in a 37 °C, 5% CO_2 incubator, rocking every 10−15 min to redistribute dilutions across the monolayer.

4.3. Prepare an agarose-MEM overlay solution by combining equal volumes of 2x Eagle's minimal essential medium (EMEM; prewarmed to 37 °C) with 1.5% agarose that has been melted in water using a microwave oven and precooled to 45 °C. Maintain the overlay solution at 42 °C using a water bath and adjust to a final concentration of 0.5 µg/mL trypsin immediately before placing on cells.

4.4. Draw off lysate dilutions from 6-well plates, then rinse cells once with 2 mL of incomplete DMEM. Gently overlay 3 mL of the agarose-MEM overlay solution onto the cell monolayer contained in each well. Allow the agarose overlay to harden at room temperature, then return plates to the incubator.

4.5. Three days later, prepare an agarose-MEM overlay solution as in step 4.3 and bring to 42 °C. Immediately prior to use, adjust the overlay solution to a final concentration of 50 µg/mL neutral red (**Table of Materials**).

4.6. Add 2 mL of the overlay solution on top of the existing agarose layer in the 6-well plates. After allowing the new agarose layer to harden, return plates to the incubator. Protect solutions and plates containing neutral red from exposure to light.

4.7. Over the next 6 h, identify rotavirus plaques in the 6-well plates with the aid of a light box. Pick clearly defined plaques using disposable transfer pipets, recovering agarose plugs that extend fully to the cell layer.

4.8. Expel the plug into a 1.5 mL tube containing 0.5 mL of DMEM incomplete medium and vortex the sample for 30 s. Amplify the plaque-isolated virus eluted into the medium by propagation on MA104 monolayers in 6-well plates or T25 flasks containing DMEM incomplete medium and 0.5 µg/mL trypsin.

NOTE: Additional information regarding isolation and titering of rotavirus by plaque assay is available in Arnold et al.[31].

5. Gel electrophoresis of viral dsRNA

5.1. Place 600 µL of clarified infected cell lysates and 400 µL of guanidinium thiocyanate in 1.5 mL microcentrifuge tubes, vortex for 30 s, and incubate at room temperature for 5 min. Add 200 µL of chloroform, vortex for 30 s, and incubate for 3 min. After centrifuging for 5 min at 13,000 x *g* (4 °C), transfer ~550 µL of the upper aqueous phase into fresh tubes.

5.2. Recover viral dsRNA from the aqueous phase by adding 2 volumes (~900 µL) of cold isopropyl alcohol and inverting tubes 4–6x. After incubating at room temperature for 10 min, centrifuge tubes for 10 min at 13,000 x *g* (4 °C). Discard supernatants and retain the RNA pellets.

5.3. Wash the pellets by adding 1 mL of 75% ethanol to the tube, inverting once, and centrifuging for 5 min at 7,500 x *g* (4 °C). After carefully removing the ethanol wash with a pipettor, allow RNA pellets to air-dry on lab bench for 5–10 min. Dissolve RNA pellets in 15 µL of nuclease-free molecular biology grade water and store at -20 °C.

5.4. Combine 10 µL of dissolved RNA samples with 2 µL of 6x DNA loading buffer, load onto precast 10% polyacrylamide mini gels (or hand-cast equivalents), and resolve RNAs by electrophoresis in Tris-glycine running buffer for 2 h under a constant current (16 mA). Soak gels for 5–10 min in water containing ~1 µg/mL ethidium bromide and detect rotavirus genome segments with a UV transilluminator.

6. Recovery and sequencing of viral dsRNA

6.1. Locate viral dsRNA bands on polyacrylamide gels preferably using low intensity long wavelength UV light, to avoid generating nucleic-acid crosslinks. Using a fresh razor blade, cut out gel fragments containing dsRNA bands and transfer into 1.5 mL microcentrifuge tubes.

6.2. After adding 10–20 µL of RNase-free water, crush each fragment with an RNase-free disposable pellet pestle designed to fit a 1.5 mL microcentrifuge tube or by drawing up and down through an 18 G needle. Incubate crushed fragments overnight at 4 °C.

6.3. Recover 3 µL of liquid from tubes containing crushed gel fragments. Generate cDNAs of the viral dsRNAs using a one-step reverse transcription polymerase chain reaction (RT-PCR) kit (**Table of Materials**) and appropriate oligonucleotide primers.

NOTE: PCR products are gel-purified with a PCR clean-up kit (**Table of Materials**) and sent out for overnight sequencing, along with primers, by commercial DNA sequence services.

7. Immunoblot analysis of viral proteins

7.1. Seed 6-well plates with 3 x 10^5 MA104 cells per well in a total volume of 2 mL of DMEM complete medium. Place plates in a 37 °C, 5% CO_2 incubator and leave until cells reach confluency (3−5 days).

NOTE: Wells with confluent monolayers will contain ~1.2 x 10^6 cells.

7.2. Treat clarified supernatants by incubating with 10 µg/mL trypsin at 37 °C for 1 h to activate recombinant rotavirus particles contained within them.

7.3. Rinse MA104 monolayers in 6-well plates 2x with 2 mL of PBS. Infect cells by adding, to each well, 200 µL of inoculum containing 3−5 plaque-forming units (PFU) per cell of trypsin-activated rotavirus in DMEM incomplete medium. Return plates to the incubator.

7.4. Every 10 min, remove plates and gently rock to redistribute inoculum across the cell monolayer. After 1 h, replace inoculum with 2 mL of DMEM incomplete medium.

7.5. At 8 h post infection, rinse cells in 6-well plates 2x with PBS. Scrape cells into 750 µL of PBS and transfer volume to a 1.5 mL microcentrifuge tube. Rinse the plate with another 750 µL of PBS and combine with the previous collected 750 µL sample.

7.6. Pellet cells in sample by centrifuging at 5,000 x *g* for 10 min at 4 °C. After discarding supernatant, store cell pellets at -80 °C until further processed.

7.7. Prepare cell lysis buffer containing 300 mM NaCl, 100 mM Tris-HCl, pH 7.4, 2% Triton X-100, and 1x EDTA-free complete protease inhibitor. After adding 300 µL of lysis buffer to frozen cell pellets, briefly vortex samples and incubate on ice for 10 min.

7.8. Repeat the process of vortexing and incubating samples on ice 3x. Afterwards, centrifuge samples at 15,000 x *g* for 10 min at 4 °C, then collect supernatants and store at -80 °C.

7.9. Resolve proteins contained in 20 µL volumes of supernatant samples by electrophoresis on precast linear 8−16% polyacrylamide mini gels and transferring to nitrocellulose membranes. Block membranes by incubating with PBS-Tween 20 (0.02%) solution containing 5% nonfat dry milk.

7.10. Probe membranes by incubating with one or more primary antibodies (e.g., guinea pig anti-NSP3 or anti-VP6 antisera, mouse monoclonal anti-Flag M2 antibody, or rabbit monoclonal anti-PCNA antibody). Detect primary antibodies by incubating membranes with horse anti-mouse IgG, anti-guinea pig IgG, or goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies, followed by the enhanced chemiluminescence (ECL) horseradish chemiluminescence substrate. Visualize luminescence signals using a gel imaging system (**Table of Materials**) or X-ray film.

8. Live-cell imaging of cells infected with FP-expressing viruses

8.1. Seed 6-well plates with 3 x 10^5 MA104 cells per well in a total of 2 mL of DMEM complete medium. Place plates in a 37 °C, 5% CO_2 incubator and leave until cells reach confluency (3−5 days).

NOTE: Wells with confluent monolayers will contain ~1.2 x 10^6 cells.

8.2. Activate rotavirus samples, of known titer, by incubation with 10 µg/mL trypsin at 37 °C for 1 h.

8.3. Rinse 6-well plates with MA104 monolayers 2x with 2 mL of PBS. Infect cells by adding to each well 200 µL of inoculum containing 3−5 PFU per cell of trypsin-activated rotavirus in DMEM incomplete medium. Return plates to the incubator and gently rock plates to redistribute inoculum across the cell monolayer every 10 min.

NOTE: Wells that are mock infected with virus-free DMEM inoculum should be included as controls.

8.4. After 1 h of virus adsorption, rinse monolayer 2x with PBS and replace medium with 0.5 mL per well of DMEM incomplete medium. At 7.5 h post infection, replace culture medium with DMEM with low background fluorescence (**Table of Materials**). At 8 h post infection, use a live cell imager to examine cells at 20x magnification for green, red, or blue fluorescence signal.

REPRESENTATIVE RESULTS:

The reverse genetics protocol described in this article proceeds through multiple distinct steps: (1) co-transfection of BHK-T7 cells with rotavirus pT7 transcription vectors and a pCMV/NP868R expression plasmid, (2) overseeding of transfected BHK-T7 cells with MA104 cells, (3) amplification of recombinant viruses present in BHK-T7/MA104 cells lysates using MA104 cells, and (4) plaque isolation of recombinant virus using MA104 cells (**Figure 2**). In our hands, the protocol is efficient, yielding titers of recombinant wildtype SA11 virus (rSA11/wt) in BHK-T7/MA104 cell lysates of ~10^4 PFU/mL and in amplified MA104 cell lysates of >1 x 10^7 PFU/mL. SA11 recombinant viruses generated by reverse genetics using modified pT7/NSP3SA11 plasmids expressing FPs (e.g., rSA11/NSP3-2A-3xFL-UnaG) grow to titers that are ~4-fold less than rSA11/wt.

Following the reverse genetics protocol, we generated recombinant SA11 viruses that were easily identified by plaque assay on MA104 cells, thus allowing plaque isolation (**Figure 3D**). Viruses in plaques were picked with a long-tip disposable transfer pipet and amplified on MA104 cells. The dsRNA genomes of plaque-purified rSA11/wt and rSA11/NSP3-2A-3xFL-UnaG viruses were extracted with guanidinium thiocyanate, resolved by electrophoresis on a 10% polyacrylamide gel, and detected by staining with ethidium bromide (**Figure 3A**). As expected, the segment 7 (NSP3) dsRNA of rSA11/NSP3-2A-3xFL-UnaG migrated much slower than that of rSA11/wt (**Figure 3A**) due to the presence of 2A-3xFL-UnaG sequences. The segment 7 (NSP3) dsRNA of rSA11/NSP3-2A-3xFL-UnaG was gel purified, converted to cDNA form by RT-PCR, and sequenced to confirm accuracy.

To check for expression of the UnaG fluorescent protein, MA104 cells in 6-well plates were infected with 3 PFU per cell of rSA11/wt and rSA11/NSP3-2A-3xFL-UnaG. At 8 h post infection, culture medium in plates were replaced with 0.5 mL of DMEM with low background fluorescence per well and the plates incubated for an additional 30 min at 37 °C in a CO_2 incubator. Afterwards, plates were examined for UnaG expression using a live cell imager. The analysis showed that rSA11/NSP3-2A-3xFL-UnaG produced green fluorescence, verifying the functionality of the UnaG gene in the recombinant virus (**Figure 3B**). In contrast, green fluorescence was not detected in cells infected with rSA11/wt. To address whether the 2A element in the modified segment 7 of rSA11/NSP3-2AxFL-UnaG promoted the expression of two separate proteins (NSP3-2A and 3xFL-UnaG), MA104 cells were infected with rSA11/NSP3-2A-3xFL-UnaG and rSA11/wt. Cell lysates were prepared from cells harvested at 8 h post infection, resolved by gel electrophoresis, and blotted onto nitrocellulose filters. The blots were probed with antibodies specific for rotavirus VP6 and NSP3, and FLAG tag. The analysis showed that NSP3-2A and 3xFL-UnaG were expressed as separate proteins in cells infected with rSA11/NSP3-2A-3xFL-UnaG, indicating that the 2A element was functional (**Figure 3C**). Cells infected with rSA11/wt did not express protein recognized by anti-FLAG antibody. The NSP3 protein present in rSA11/NSP3-2A-3xFL-UnaG-infected cells by anti-NSP3 antibody migrated slightly slower than NSP3 present in rSA11/wt-infected cells due to the presence of remnant 2A residues at the C-terminus of NSP3.

FIGURE LEGENDS:

Figure 1: Rotavirus reverse genetics plasmids. (**A**) Full-length cDNAs of the 11 SA11 rotavirus genome segments are positioned within pT7 plasmids, ligated upstream with a promoter for T7 RNA polymerase and downstream with an HDV ribozyme. In the presence of T7 RNA polymerase, the rotavirus pT7 plasmids produce full-length SA11 (+)RNAs with authentic 5' and 3' termini. (**B**) Schematics of the (+)RNA and protein products made by the pT7/NSP3-2A-3xFL-UnaG plasmid. The schematic includes the NSP3 cDNA sequence, and sequences for the porcine teschovirus 2A-like (2A) element, the 3x FLAG (FL) tag, and the green fluorescent protein UnaG. The position of the 2A translational stop-restart site is indicated with a red arrow. Due to the activity of the 2A element, translation of the RNA produces two proteins. The NSP3 portion contains remnants of the 2A element and the UnaG portion is fused to a 3x FLAG tag.

Figure 2: Rotavirus reverse genetics system. BHK-T7 monolayers are transfected with 11 pT7 plasmids, each expressing a different SA11 (+)RNA, and a pCMV vector expressing the African swine fever virus (ASFV) NP868R capping enzyme (pCMV/NP868R). At 3 days post infection (d.p.i.), the BHK-T7 cells are overseeded with MA104 cells. At 7 days post infection, recombinant rotaviruses in BHK-T7/MA104 cell lysates are amplified by passage on MA104 cells, then isolated by plaque purification.

Figure 3: Characteristics of the recombinant strains rSA11/wt and rSA11/NSP3-2A-3xFL-UnaG. (**A**) Electrophoretic profiles of the dsRNA genome segments of plaque-isolated rSA11 strains. The 11 genome segments are numbered and the shift in the position of segment 7 (NSP3) is indicated with a red line. (**B**) Fluorescence detected in rSA11-infected MA104 cells at 8 h post infection using a live cell imager (20x magnification) set on the green detection channel. Scale bar = 100 µm. (**C**) Immunoblot analysis of proteins present at 8 h post infection in MA104 cells infected with rSA11 strains using guinea pig anti-VP6 and anti-NSP3 antisera and mouse anti-FLAG monoclonal antibody. (**D**) Plaques produced by rSA11/wt on MA104 cells at 3 days post infection and detected by neutral red staining.

DISCUSSION:

In our laboratory, we routinely rely on the reverse genetics protocol described herein to produce recombinant SA11 rotaviruses. With this approach, individuals with little experience in molecular biology techniques or working with rotaviruses recover recombinant viruses even on their first attempt. We have generated close to 100 recombinant viruses following this protocol, including those with genomes that have been re-engineered to express foreign proteins (e.g., FPs) and that contain sequence additions, deletions, and point mutations.

The conditions and incubation times given in this protocol apply to the recovery of well growing strains of recombinant viruses. Adjustments should be considered if attempting to recover SA11 viruses that, due to genetic modification, may be expected to grow poorly. In particular, because the titer of such viruses in transfected BHK-T7/MA014 cell lysates may be low, we typically double the amount of lysate used as inoculum in subsequent amplification steps. Moreover, at the amplification step, poorly growing viruses may require longer times of incubation before reaching levels of CPE sufficient for cell harvesting. Indeed, with such viruses, we may allow infections to proceed for 10−14 days, or even longer, before harvesting cells. Finally, poorly growing viruses are likely to generate small, slow-growing plaques on MA104 cells. Thus, to plaque isolate these viruses, it may be necessary to allow plaques to develop until 6−10 days post infection before staining cells with neutral red and picking plaques.

In our experience, the single most important factor in the reliable recovery of the recombinant rotavirus is the use of healthy, well-maintained BHK-T7 cells. In our laboratory, we routinely passage BHK-T7 cells 2x a week at the same dilution using medium supplemented not only with 10% FBS, but also with NEAA, TPB, and high levels of glucose (GMEM complete medium). The additional supplements help BHK-T7 cells retain extended viability following plasmid transfection, a factor likely crucial for recovering poorly growing mutant recombinant viruses. We supplement the medium every other passage with G418, an antibiotic which selects for

maintenance of the T7 polymerase expression plasmid. Passage conditions should be such that BHK-T7 cells are never allowed to grow past confluency, conditions that rapidly lead to decreased cell viability. For us, BHK-T7 cells that have been allowed to overgrow perform poorly in the reverse genetics protocol, even if the cells are subsequently passaged appropriately multiple times. Instead of attempting to rehabilitate overgrown BHK-T7 cells, we restart the lineage with cells previously stored in liquid nitrogen.

Mycoplasma contamination of BHK-T7 and MA104 cells can be a major factor in the failure of the rotavirus reverse genetics system to generate recombinant virus. In our laboratory, we use a PCR-based mycoplasma detection kit (**Table of Materials**) to check for contamination of cell lines, and when detected, it is most often associated with our BHK-T7 cells. We have not attempted to cure cell lines of contaminating mycoplasma, instead re-establishing the lines with earlier mycoplasma-free passages stored in liquid nitrogen. Prior to starting new cell lines, we discard all previously used medium and medium supplements and thoroughly decontaminate incubators, biological safety cabinets, water baths, lab benches, and pipettors. We also use mycoplasma detection kits to check stocks of recombinant viruses for contamination. Because of the resistance of the rotavirus particles to denaturation by organic solvents, such as Vertrel VF[32], it is possible to free virus stocks from contaminating mycoplasma, negating the need to regenerate recombinant viruses by reverse genetics. It is important to stress that cell lines and virus preparations received into the laboratory should be checked for mycoplasma contamination prior to routine use.

Although day-in and day-out, we use the same protocol to generate recombinant viruses, we know that certain modifications can be made which will not preclude virus recovery. For example, (i) co-transfection of the pCMV/NSP868R capping-enzyme plasmid with SA11 pT7 vectors is not required for recovery of recombinant viruses. While addition of the capping plasmid yields higher virus titers in transfected BHK-T7/MA104 cell lysates, we have been able to recover numerous viruses, including those expressing FPs, without it. However, we have concluded that expression of the capping enzyme by pCMV/NP868R may contribute significantly to the recovery of less fit viruses. (ii) We have found that recombinant viruses can be generated even if the amount of the transfection reagent (**Table of Materials**) used in the reverse genetics protocol is reduced by one-half, a modification that can significantly reduce expenses. (iii) Similarly, we have determined that M199 complete medium can be used in place of DMEM complete medium. (iv) Finally, there is no set requirement concerning the type of vector backbone that must be used in producing SA11 T7 transcription vectors. As long as the viral cDNA in the plasmid is surrounded by an upstream T7 promoter and a downstream HDV ribozyme and T7 terminator, the plasmid can be expected to support the recovery of recombinant virus. Notably, pGEM-, pBluescript, and pUC-based vectors have been used successfully in the reverse genetics system.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

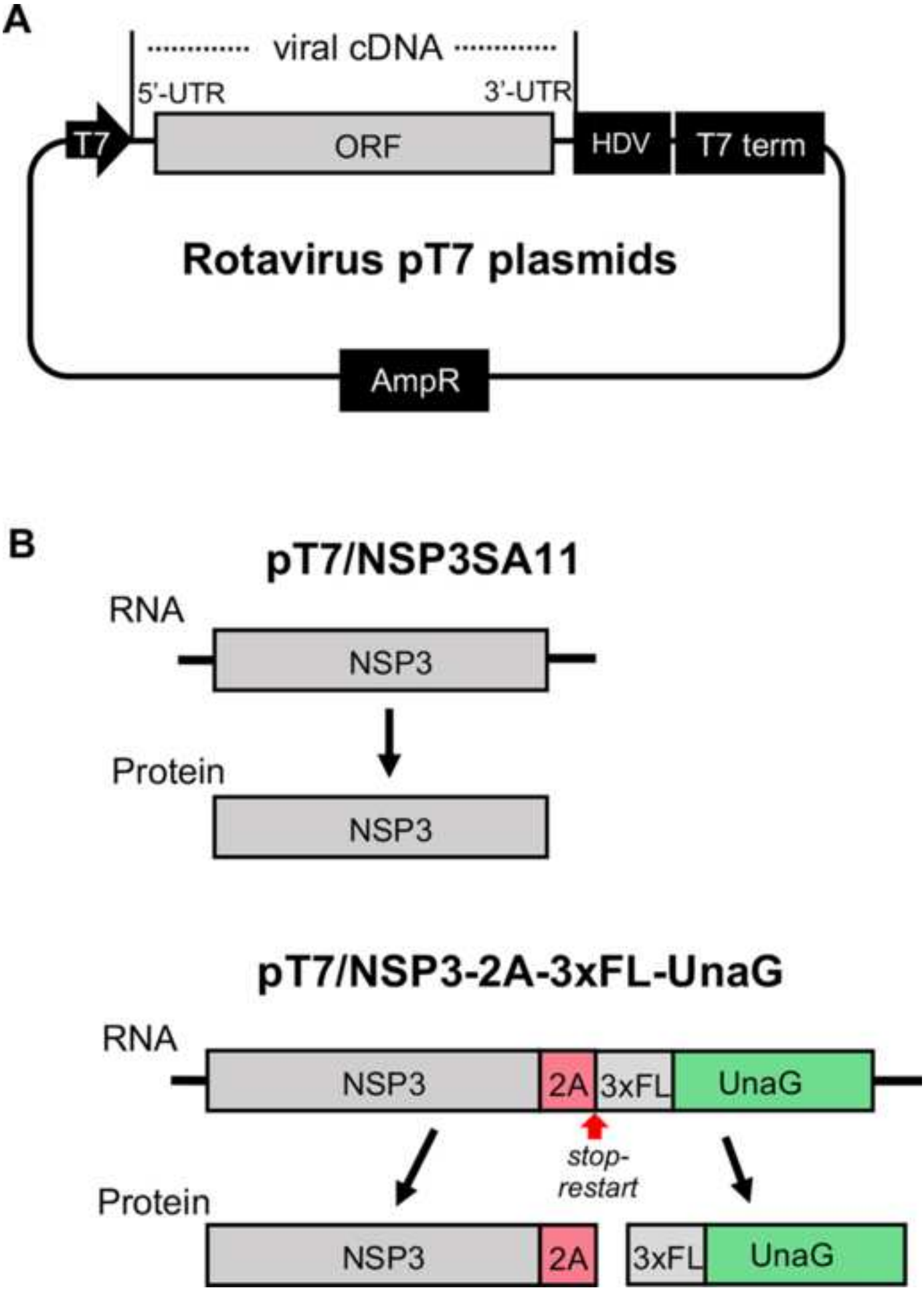

Figure 2

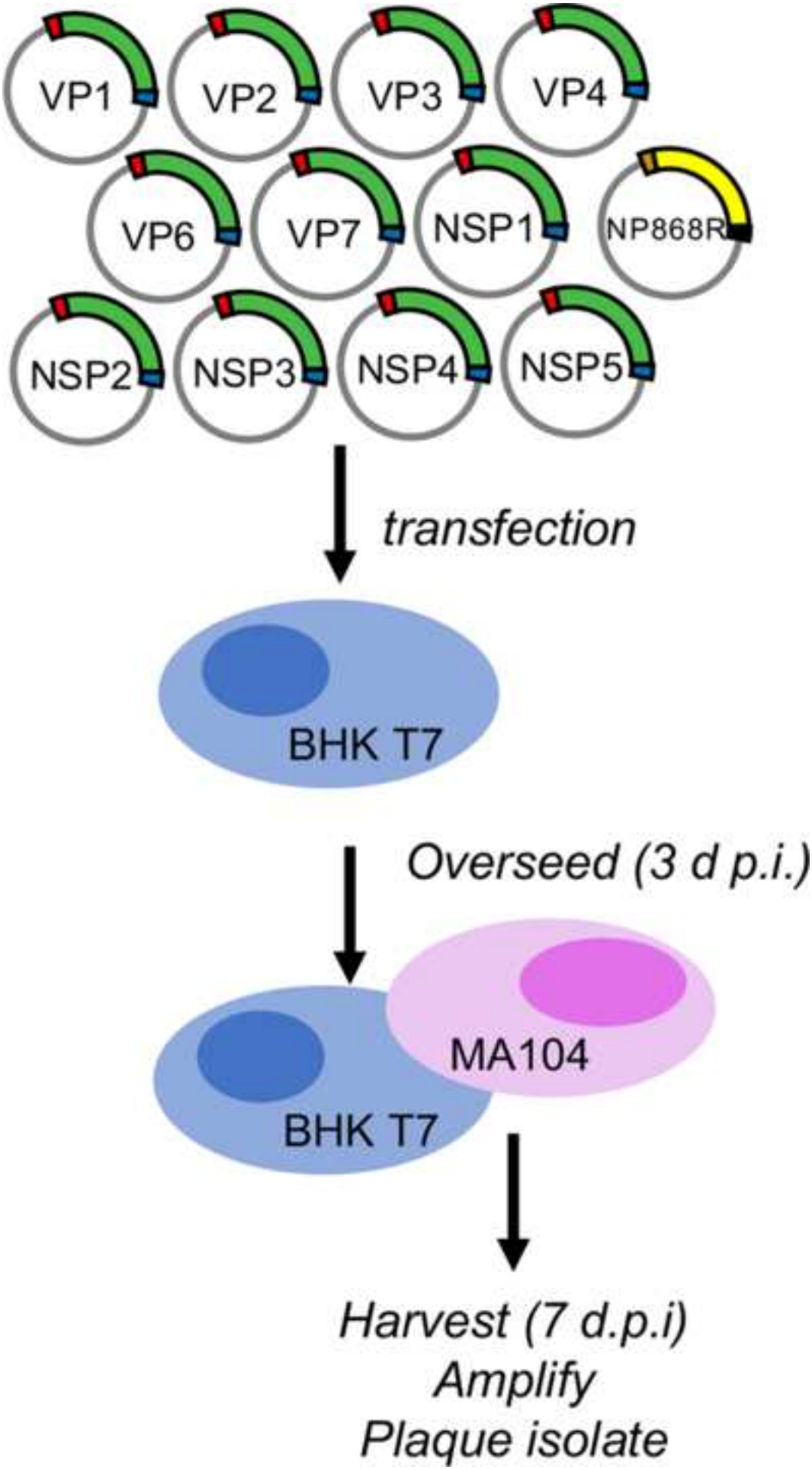

Figure 3

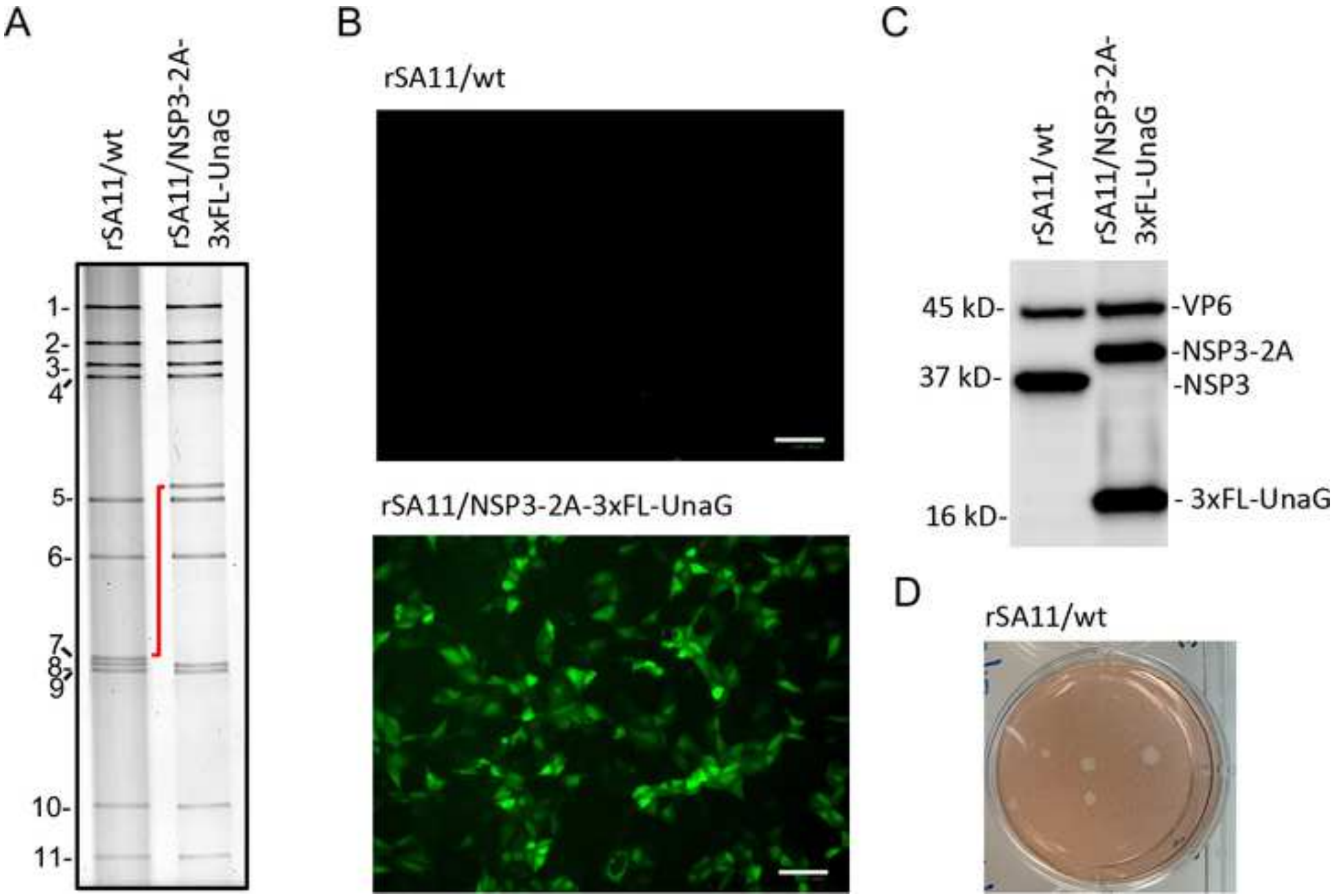

Table of Materials

Click here to access/download;Table of Materials;61039_2019JoVE_Materials_Revisedv3.xlsx

Name of Material/ Equipment	Company	Catalog Number
Baby Hamster Kidney - T7 RdRP (BHK-T7) Cells		
Bio-Rad 8-16% Tris-Glycine Polyacrylamide Mini-Gel	Bio-Rad	45608105
Cellometer AutoT4 viable cell counter	Nexcelom	
ChemiDoc MP Gel Imaging System	Bio-Rad	
Chloroform	MP	194002
Clarity Western Enhanced Chemiluminescence (ECL) Substrate	Bio-Rad	170-5060
Competent E.coli DH5alpha Bacteria	Lucigen	60602-2
Complete Protease Inhibitor	Pierce	A32965
Disposable Transfer Pipettes, Ultrafine Extended Tips	MTC Bio	P4113-11
Dulbecco's Modified Eagle Medium (DMEM)	Lonza	12-604F
Eagle's Minimal Essential Medium, 2x (2xEMEM)	Quality Biological	115-073-101
Ethanol, Absolute (200 proof)	Fisher Bioreagents	BP2818-500
Ethidium Bromide Solution (10 mg/ml)	Invitrogen	15585-011
Fetal Bovine Serum (FBS)	Corning	35-010-CV
Fetal Bovine Serum (FBS), Heat Inactivated	Corning	35-011-CV
Flag M2 Antibody, Mouse Monoclonal	Sigma-Aldrich	F1804
GenEluate HP Plasmid Midiprep Kit	Sigma	NA0200-1KT
Geneticin (G-418)	Invitrogen	10131-027
Gibco FluroBrite DMEM	ThermoFisher	A1896701
Glasgow Minimal Essential Medium (GMEM)	Gibco	11710-035
Goat Anti-Rabbit IgG, Horseradish Peroxidase (HRP) Conjugated	Cell-Signaling Technology	7074S
Guinea Pig Anti-NSP3 Antiserum	Patton lab	lot 55068
Guinea Pig Anti-VP6 Antierum	Patton lab	lot 53963
Conjugated	KPL	5220-0366
Horse Anti-Mouse IgG, Horseradish eroxidase (HRP) Conjugated	Cell-Signaling Technology	7076S
iNtRON Biotechnology e-Myco Mycoplasma PCR Detection Kit	JH Science	25235
Isopropyl alcohol	Macron	3032-02
L-glutamine Solution (100x)	Gibco	25030-081
Luria Agar Powder (Miller's LB Agar)	RPI research products	L24020-2000.0
Medium 199 (M199) Culture Medium	Hyclone	Sh30253.01

Minimal Essential Medium -Eagle Joklik's Forumation (SMEM)	Lonza	04-719Q
Monkey Kidney (MA104) Cells	ATCC	ATCC CRL-2378.1
NanoDrop One Spectrophotometer	ThermoScientific	
Neutral Red Solution (0.33%)	Sigma-Aldrich	N2889-100ml
Non-Essential Amino Acid Solution (100x)	Gibco	11140-050
Novex 10% Tris-Glycine Polyacrylamide Mini-Gel	Invitrogen	XP00102BOX
Nuclease-Free Molecular Biology Grade Water	Invitrogen	10977-015
NucleoSpin Gel and PCR Clean-Up Kit	Takara	740609.25
Opti-MEM Reduced Serum Medium	Gibco	31985-070
Pellet pestle (RNase-free, disposable)	Fisher	12-141-368
Penicillin-Streptomycin Solution, (100x penn-strep)	Corning	30-002-Cl
Phosphate Buffered Saline (PBS), 10x	Fisher Bioreagents	BP399-20
Porcine Trypsin, Type IX-S	Sigma-Aldrich	T0303
PureYield Plasmid Miniprep System	Promega	A1223
Qiagen Plasmid Maxi Kit	Qiagen	12162
Qiagen Plasmid Midi Kit	Qiagen	12143
QIAprep Spin Miniprep Kit	Qiagen	27104
SA11 pT7 Transcription Vectors	Addgene	89162-89172
SA11 pT7/NSP3 Transcription Vectors Expressing Fluorescent		
SeaKem LE Agarose	Lonza	50000
SeaPlaque agarose	Lonza	50100
Superscript III One-Step RT-PCR kit	Invitrogen	12574-035
Trans-Blot Turbo Nitrocellulose Transfer Kit	Bio-Rad	170-4270
Trans-Llot Turbo Transfer System	Bio-Rad	
TransIT-LTI Transfection Reagent	Mirus	MIR2306
Tris-Glycine-SDS Gel Running Buffer (10x)	Bio-Rad	161-0772
Triton X 100	Fisher Bioreagents	BP151-500
Trizol RNA Extraction Reagent	Ambion	15596026
Trypan blue	Corning	25-900-CI
Trypsin (0.05%)-EDTA (0.1%) Cell Dissociation Solution	Quality Biological	118-087-721
Tryptose Phosphate Broth	Gibco	18050-039
Tween-20	VWR	0777-1L

Vertrel VF solvent	Zoro	G0707178
Zoe Fluorescent Live Cell Imager	Bio-Rad	

Comments/Description

Contact: ubuchholz@niaid.nih.gov

DMEM with low background fluorescence

Contact: jtpatton@iu.edu
For gel electrophoresis
For plaque assay

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have proofread manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have modified portion of the protocol used in preparing the video to include sufficient detail to supplement actions.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

We have adjusted the numbering throughout to confirm with JoVe instructions.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted in yellow portion of manuscript to be visualized (see pages 5-8).

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and

in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have made modifications and additions to the Discussion that appropriately address the methods and protocols addressed in the manuscript.

• **Figures:** Add scale bars to fig 3 B.

We have added a scale bar.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The figures and tables are all new and have not been previously published.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:

General Comments:
At the International Symposium on dsRNA Viruses in Houffalize about a year ago scientists found it desirable to have a standardized procedure of the production of recombinant species A rotaviruses (RVA) with a high likelihood of success, even in the hands of novices in RV research. Here such a procedure has been described in detail, and the reliability of this and very similar procedures has already been demonstrated by a number of laboratories involved in RVA research. The description has been very meticulously described, and this reviewer has only a few, relatively minor Specific Comments. On the whole, this is a very important contribution to the applicability of reverse genetics to rotavirus research and is, in this reviewer's view, eagerly awaited by the scientific community.

Specific Comments:

Line:

2 Reconsider slight variation in Title, e.g. 'Rescue of recombinant rotavirus able to express fluorescent reporter and other heterologous proteins', or similar.

Thank you. Both reviewers 1 and 3 suggested modification of the title, which we have done.

28 Consider phrasing: … in infants and young children and in the young of many mammalian and avian host species. [in analogy also in line 45]

Changes have been made in both places of the manuscript in keeping with the reviewer's suggestion.

33 … expressing full length rotavirus (+)RNAs…

Change was made.

45 … As members of the Reoviridae family, ….

Change was made.

53 … rotavirus-associated childhood deaths has decreased from…

Change was made.

76 … three HCMV promoter-driven expression plasmids…

Change was made.

77 … and two encoding the subunits of the vaccinia ….

Change was made.

82 … for the viral factory (viroplasms) building blocks…

Change was made.

101 … FP ORF23, 25, 26. Through this…

Citations were corrected.

103 … (yellow)23, 28. These…

Citations were corrected.

130 Indicate supplier and specific preparation of trypsin used. [porcine pancreatic trypsin type IX??]

Supplier and catalog number included in Table of Materials.

154 … purification kit, following manufacturer's instructions. Larger…

Change was made.

158 … to be endo-free. Please clarify.

Sentence was rephrased to clarify.

236 Indicate supplier and preparation of agarose used. [Some agarose preparations can hinder plaque formation.]

Supplier and catalog number included in Table of Materials

255 NOTE: Additional information detailing isolation… is available…

Change was made.

273 … Soak gels… in water… Please confirm. Or was the running buffer used?

Water was used, as indicated in manuscript.

286 … PCR products are gel-purified… mention kit or procedure. …. and sequenced. [mention procedure or reference].

Requested information was provided.

298 … DMEM…

Change was made.

376 … recognized by anti-Flag antibody (data not shown). The NSP3 protein…

Text was left as is. Figure 3C does show that rSA11/wt did not produce FLAG-tagged protein.

382 to 407. Figs. 1-3 are of suboptimal quality and could be improved.

The images in the manuscript for review were provided at 72 dpi. The quality will be greater with the 300 dpi images to be published.

383 … are ligated upstream to a promoter for T7 RNA polymerase and downstream to a HDV ribozyme…

Change was made.

404 … The green bar is equal… The green bar is not shown.

We have modified figure to make the size bar more obvious.

406 … with rSA11 strains. (D) Plaques produced…

Change was made.

430 Since the condition of BHK-T7 cells is crucial for success, this topic should be slightly expanded, and availability be identified in the Table of Materials.

*We have included additional comments regarding BHK-T7 cells in the Discussion and commented on their availability in the **Protocol** section (section 1.1.)*

445 … without it. However, we concluded that…

Change was made.

455 Consider providing refs for pGEM- and pUC-based vectors.

Information provided.

527 Will ref [25] be submitted in the near future? It should not be cited as ms 'in preparation'.

It will be submitted within the next 10 days so we have changed to 'submitted'.

537f The Table of Materials is very important and should be mentioned in the Text as Table 1.

*We have added 'Tables of Materials' to the **Protocol** section (section 1.2)*

Reviewer #2:

Manuscript Summary:
Nicely written protocol describing a modified version of the rotavirus RG protocol using the increased amounts of NSP2 and NSP5 plasmids and the ASFV capping enzyme NP868R. The text is clear, well written and easy to follow. I could see no issues. The discussion nicely points out the importance of careful passage of BHK-T7 cells and modifications that can be made to recover less fit recombinant viruses. This protocol will be useful to workers in the field.

Major Concerns:
None
Thanks!

Reviewer #3:

Manuscript Summary:

This is a timely protocol that is very much needed by the rotavirus community. Several laboratories are still struggling with this methodology to have a reproducible recovery of rotavirus reassortants and mutants, and the methods described in this protocol will be very helpful.

In general, the method is a detailed and simplified method from the original system described by Kanai et al 2017, in which three of the support plasmids included in the original protocol, were either eliminated (pFAST), or substituted by one CMV vector that encodes a capping enzyme from ASFV which replaces two plasmids that encoded the vaccinia virus D1R-D1L.

The protocol described here is carefully written and guides step by step through the complete rescue system. I only have minor comments:

1.- I think that the title needs to include the words REVERSE GENETICS in it, to emphasize, for the non-rotavirus people, the fact that this system is not a simple mutagenesis method for rotaviruses, something like:
"Simplified reverse genetic method to recover recombinant rotaviruses expressing reporter proteins".

We have made the suggested change.

2.- Page 2, line 87, needs to cite the work by Duarte et al, JVI 2019:93 e01739-18, where they report the use of reverse genetics to make NSP3 chimeras.

Thanks, we have added the missing reference.

3.-Page 3, line 109, and Table of Materials: The source of BHK-T7 cells needs to be cited.

We have added information regarding the source of BHK-T7 cells to ***Protocol****, section 1.1.*

4.- Page 4 line 157. In our hands, it is essential to use endo-free kits for plasmid purification. Maybe you should be more cautious with this comment because the quality of other reagents used in plasmid purification in other places might be different from your those in your place.

We appreciate the point, but the cost of purchasing endo-free plasmid purifications kits is very high. We were trying to emphasize that we have found that it is not necessary to use endo-free kits. In addition, we know that other labs routinely using rotavirus reverse genetics system are not using endo-free kits either. As a result, we have left the text as is.

5.- Page 4, line 174, overnight needs to be deleted if the cells are incubated for at least 2 days.

Change was made.

6.- Page 5, line 206, …by adding 3 ??? of a 0.2 mg…. MICROLITERS????

Correction was made.

7.- Page 7, Just a curiosity, is it necessary to cut the band from a gel to do the RT-PCR-sequencing? Why not RT-PCR directly from total RNA from the isolated plaque?

Because of genetic instability, in some cases, viruses are generated that contain segments with aberrant genome segments. To assure sequencing of segments that are not aberrant, we gel purify RNAs that are appropriately sized. (Frequently, we also isolate and sequence aberrant RNAs to gain a sense of how genetic instability affects the sequence and coding potential of the RNA).

8.- Page 8, line 345 and in general. Why naming the recombinant wtSA11, as rSA11/NSP3 instead of simply rSA11? I think it is confuse to add NSP3 in the wt recombinant virus if this gene does not have modifications.

Reviewer is correct. We have changed rSA11/NSP3 to rSA11/wt.

9.- In various parts of the protocol, Novex gels are mentioned, I think this is not essential. Why not just mention SDS- X% acrylamide gels?

In the text, we do provide information on the % of the polyacrylamide gels that we are using and point out that instead of precast Novex gels, one can use hand-cast gels. However, it is our experience that the precast gels give better resolution and are more reliable in analyzing RNAs, so we continue to mention them in the manuscript.