

## Reverse Genetics of Newcastle Disease Virus

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### Abstract

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

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

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## 1 Introduction

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

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

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

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

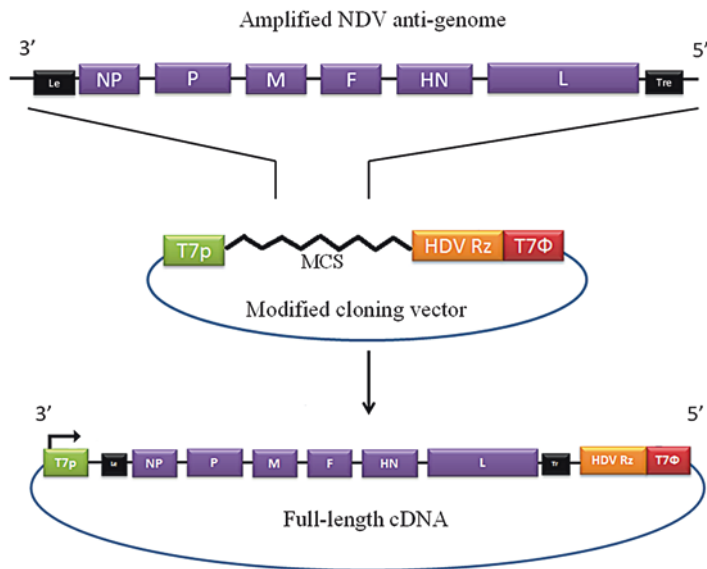
The development of reverse genetics for NDV has allowed the genetic manipulation of its genome to achieve a better understanding of viral functions during replication and infection [11–16]. NDV reverse genetics has made possible the development of a valuable recombinant vaccine system, enabling expression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

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

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

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

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



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

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

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

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## 2 Materials

### 2.1 NDV Genome Manipulation

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

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

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

### 2.1.2 NDV Gene Replacement

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

**2.2 Cell Culture  
Growth  
and Maintenance**

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

**2.3 Infection-  
Transfection**

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

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

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

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

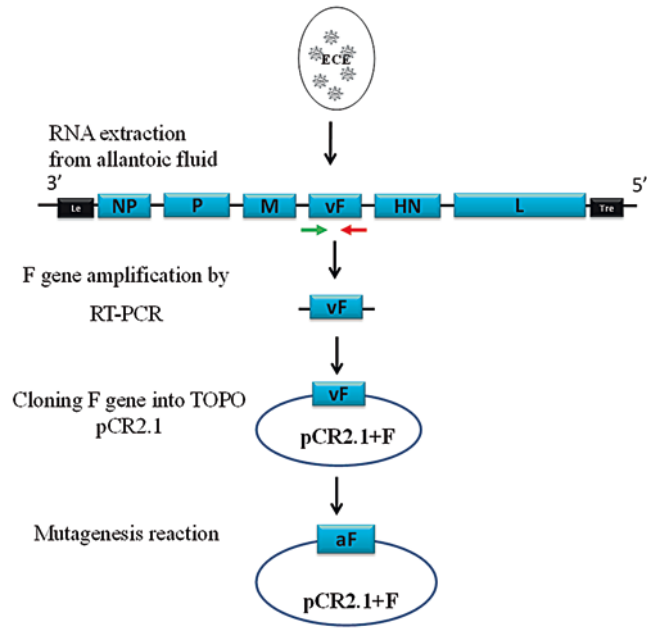
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### **3 Methods**

#### **3.1 NDV Genome Manipulation**

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

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



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

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

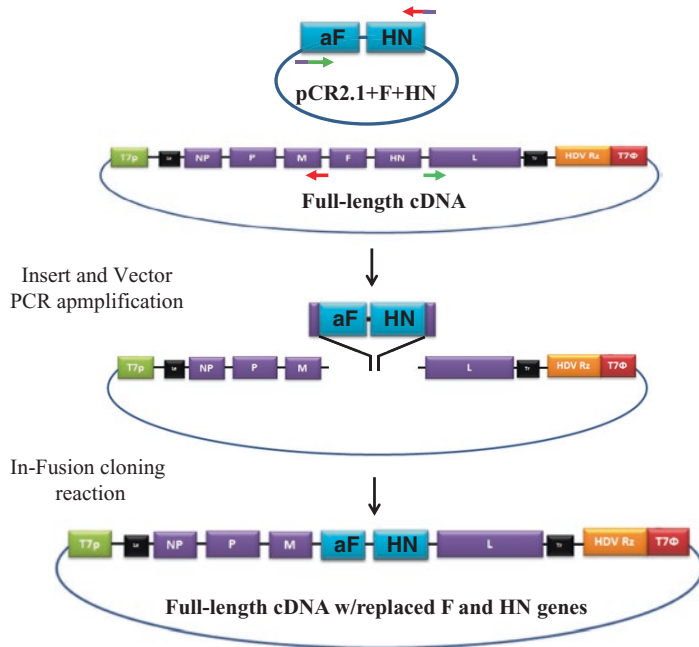


mutagenic sequence has to be flanked by 10–15 perfectly matched nucleotides on each side, and the annealing temperatures should fall between 65 and 72 °C (refer to the kit's manual for details on primer design; i.e., forward 5'-ATC TGG AGG GgG GAG ACA Ggg ACG CcT TAT AGG TGC CG-3', reverse 5'-GTG GAC ACG GAC CCT TGT ATC CTA CGG ATA GAA TCG CCC-3').

- (c) Perform the PCR reactions as directed in the mutagenesis kit using the fusion-gene plasmid. This reaction will amplify the full plasmid and yield a linearized product.
- (d) Analyze the mutagenesis PCR product through gel electrophoresis, excise the band showing the expected size, and perform DNA gel extraction.
- (e) To circularize the plasmid contained in the mutagenesis PCR product, use the T4 DNA ligase provided with the mutagenesis kit and follow the manufacturer's instructions.
- (f) Transform circularized plasmid into TOP10 chemically competent cells following **steps 6–13** from this section.
- (g) Confirm the sequence of the mutated fusion gene through sequencing analysis (*see Note 2*).

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

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



**Fig. 3** Gene replacement

- Analyze the PCR products for both vector and insert through gel electrophoresis, excise the bands showing the appropriate size, and gel purify the products.
- Quantify the DNA concentrations to ensure that there is enough DNA for the cloning reactions, which will require between 50 and 200 ng of vector and insert, respectively. It may be necessary to prepare more than one PCR reaction for vector amplification in order reach the required DNA concentration (*see Note 3*).
- Prepare the cloning reactions in 10–20  $\mu\text{L}$  final volume (depending on the vector and insert concentrations).
- Place reactions in a thermocycler, incubate for 15 min at 50  $^{\circ}\text{C}$ , and then place reactions on ice. At this point, the cloning reactions can be stored at  $-20^{\circ}\text{C}$  or can be used to transform competent cells.
- Proceed to transform the cloning reactions into Max Efficiency<sup>®</sup> Stab2<sup>™</sup> competent *E. coli*, following the manufacturer's instructions.
- Pre-warm the LB plates containing 100  $\mu\text{g}/\text{mL}$  of ampicillin (or the required antibiotic).
- Plate 100  $\mu\text{L}$  of the transformed *E. coli* suspension on LB plates and incubate overnight at 30  $^{\circ}\text{C}$  (between 16 and 24 h).

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

### **3.2 Cell Culture Growth and Maintenance**

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

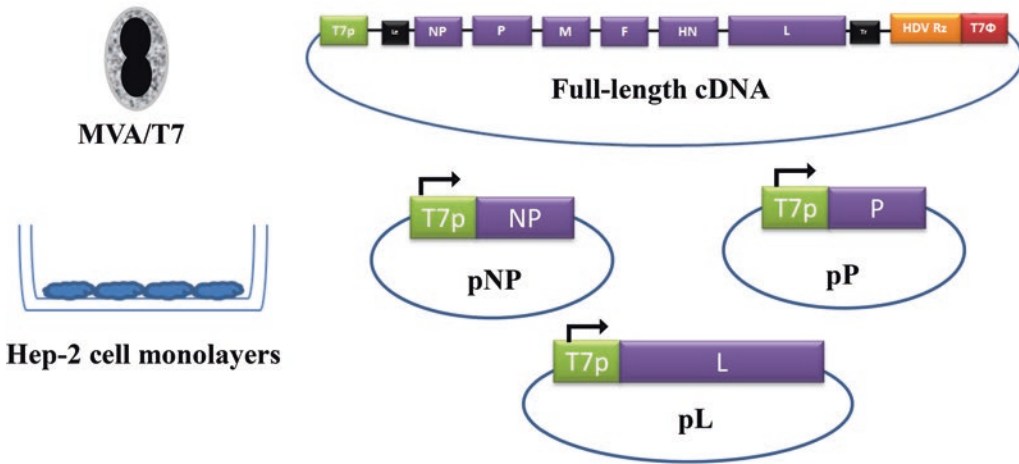
1. After extracting a vial of cells from the liquid nitrogen, allow them to thaw.
2. Dispense the contents of the vial into a T25 flask containing pre-warmed DMEM supplemented with 10% FBS and 1 $\times$  penicillin/streptomycin solution (complete media).
3. Place the cells into a cell culture incubator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

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

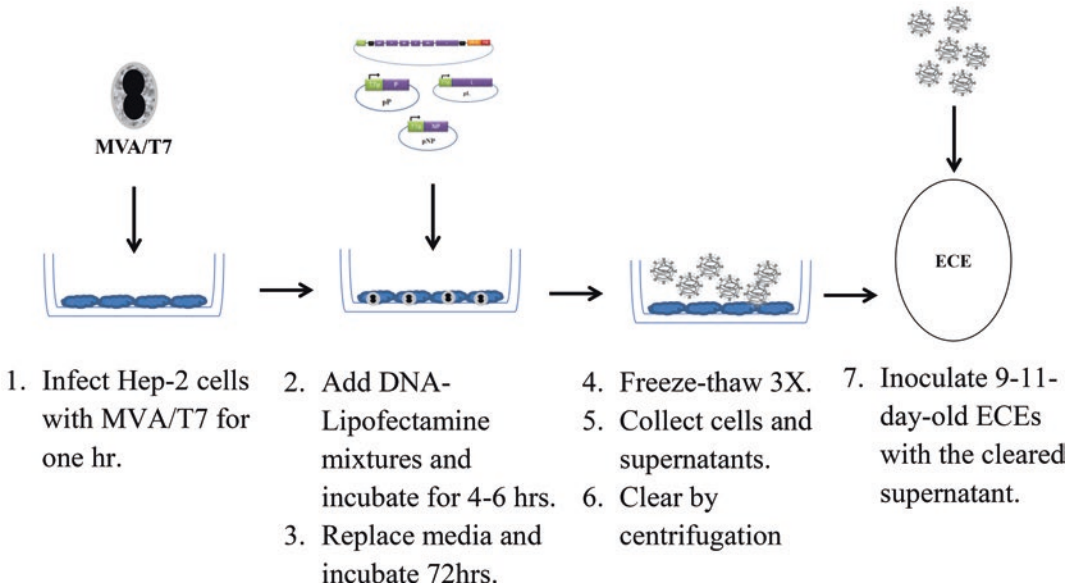
**3.3 Rescue  
of Lentogenic  
Newcastle Disease  
Viruses (Fig. 4)**

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

### A. Elements



### B. Procedure



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

10. Add 1 mL of fresh Opti-MEM to each well and the DNA-Lipofectamine mixtures in a dropwise fashion to the corresponding wells.
11. Gently rock the plates to evenly distribute the mixtures.
12. Incubate during 4–6 h at 37 °C under a 5% of CO<sub>2</sub> atmosphere.

13. Following cell incubation replace the old media with fresh DMEM supplemented with 1× penicillin/streptomycin solution and porcine pancreatic trypsin (1 µg/mL). *Do not add FBS to the media.*
14. Incubate for 72 h at 37 °C under a 5% CO<sub>2</sub> atmosphere, checking cells daily for cytopathogenic effects.
15. After 72 h, harvest the cells and perform three rapid freeze-and-thaw cycles. This can be achieved using an ultra-freezer and the cell culture incubator.
16. Clear the cell culture supernatants by centrifugation at 1200 × *g* for 10 min at 4 °C.
17. Transfer the cleared supernatants into 1.5 mL microcentrifuge tubes and set on ice for ECE inoculation.

**3.4 Rescue of Mesogenic and Velogenic Newcastle Disease Viruses**

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

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

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

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

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## 4 Notes

### 1. *Safety considerations*

- (a) According to the Code of the Federal Regulations, Title 9, Chapter I, Sub-chapter E, Part 121.3, nucleic acids that can produce infectious forms of any select agent are subjected to the regulations for select agents. Therefore, these experiments should be conducted in a BSL-3 facility at all times, until rescued viruses are deselected.
- (b) Wear personal protective equipment: lab coat or disposable gowns, safety glasses, and gloves.
- (c) Hep-2 cells contain human papillomavirus; therefore, they should be grown and maintained under BSL-2 conditions (refer to the ATCC website for details).
- (d) Conduct all cell culture work and virus rescue procedures in a biosafety cabinet in order to maintain sterility conditions and reduce pathogen exposure.

### 2. *Considerations related to genome size*

- (a) Always sequence after each step involving RT-PCR, PCR amplification, or any genetic manipulation of the genome. It is important to confirm that there are no unexpected mutations or deletions, that the intentional mutations were introduced, and that the insert or gene replacements are in the correct location and orientation.
- (b) *The rule of six*: Newcastle disease virus follows the so-called rule of six, which refers to the fact that the NDV genome's length is always a multiple of six. This has to do with the encapsidation process, where the ribonucleoprotein complex molecules bind to six nucleotides at a time. If, for any reason, the length of the genome is not a multiple of six, there is no proper encapsidation and therefore the ability to rescue viable viruses is hampered [42]. This is an important consideration that has to be taken into account during the design and development of the full-length cDNA plasmids.
- (c) *Size of the insert*: It has been reported that insertion of nucleic acids that increase the size of the NDV genome may attenuate the virus, probably by decreasing its replication ability [21, 43]. In addition, due to its non-segmented



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

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

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

- (a) The use of pfuULTRA™ II Fusion HS DNA polymerase (Stratagene) to amplify the vector/insert before cloning to generate the full-length cDNA plasmid, is recommended since other PCR kits may not produce a linearized vector/insert of enough quality for the In-Fusion cloning technique.
- (b) When amplifying/linearizing the vector before cloning of the full-length cDNA plasmid, one may need to prepare multiple PCR reactions to obtain enough DNA for the cloning step. After the DNA gel purification step, multiple vector PCR products can be concentrated into a single tube using a PCR purification kit or through ethanol precipitation.

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