# Chapter 9

# Generation of a Single-Cycle Replicable Rift Valley Fever Vaccine

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## Abstract

Rift Valley fever virus (RVFV) (genus *Phlebovirus*, family *Bunyaviridae*) is an arbovirus that causes severe disease in humans and livestock in sub-Saharan African countries. The virus carries a tripartite, single-stranded, and negative–sense RNA genome, designated as L, M, and S RNAs. RVFV spread can be prevented by the effective vaccination of animals and humans. Although the MP-12 strain of RVFV is a live attenuated vaccine candidate, MP-12 showed neuroinvasiveness and neurovirulence in young mice and immunodeficiency mice. Hence, there is a concern for the use of MP-12 to certain individuals, especially those that are immunocompromised. To improve MP-12 safety, we have generated a single-cycle, replicable MP-12 (scMP-12), which carries L RNA, S RNA encoding green fluorescent protein in place of a viral nonstructural protein NSs, and an M RNA encoding a mutant envelope protein lacking an endoplasmic reticulum retrieval signal and defective for membrane fusion function. The scMP-12 undergoes efficient amplification in the Vero-G cell line, which is a Vero cell line stably expressing viral envelope proteins, while it undergoes single-cycle replication in naïve cells and completely lacks neurovirulence in suckling mice after intracranial inoculation. A single-dose vaccination of mice with scMP-12 confers protective immunity. Thus, scMP-12 represents a new, promising RVF vaccine candidate. Here we describe protocols for scMP-12 generation by using a reverse genetics system, establishment of Vero-G cells, and titration of scMP-12 in Vero-G cells.

**Key words** Rift Valley fever virus, Vaccine, Single-cycle replicable virus, Reverse genetics, Transfection, Stable cell establishment

## 1 Introduction

Rift Valley fever virus (RVFV), a member of the genus Phlebovirus within the family Bunyaviridae, carries a tripartite, single-stranded, and negative-sense RNA genome [1–3]. The L RNA encodes the L protein, a viral RNA-dependent RNA polymerase. The M RNA encodes four proteins, including two accessory proteins, the NSm and 78 kDa proteins, and the two major viral envelope proteins Gn and Gc (Gn/Gc). The S RNA uses an ambisense strategy to express nucleocapsid (N) protein and an accessory protein, NSs. In infected cells, L and N proteins drive viral RNA synthesis in the cytoplasm,

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while viral assembly and budding take place at the Golgi apparatus where Gn/Gc accumulates.

The virus is transmitted by mosquitoes and maintained by transovarial transmission in local mosquitoes in sub-Saharan Africa. The transmission of the virus among domestic ruminants has resulted in a high mortality rate and spontaneous abortions in virtually all pregnant animals [1]. Human infection occurs via RVFV-infected mosquito bites or direct transmission of the virus from infected animal tissues or blood. Human disease symptoms include febrile illness, retinitis, encephalitis, and, in about 1 % of cases, hemorrhagic fever [4–6]. Since RVFV is able to infect various species of mosquitoes [7], it has the potential to spread to other areas of the world by movement of infected vectors. Indeed, RVFV has already spread outside of the African continent, e.g., to the Arabian Peninsula. There is an increasing consensus that the spread of Rift Valley fever into North America and Europe is no longer a question of if, but when. RVFV has also been considered a potentially exploitable agent for bioterrorism [8].

RVFV spread can be prevented by the effective vaccination of animals and humans [9]. RVFV is considered to be serologically monotypic [10–12]. Also, humoral immunity, particularly neutralizing antibodies that recognize Gn/Gc, is important for protection [13–21]. Therefore, RVFV vaccines that elicit strong humoral immune responses will be able to prevent infection by any strain of RVFV. Although a good human RVFV vaccine is urgently needed, there is no approved vaccine that can be adapted to massive vaccination programs. An attenuated RVFV, MP-12, was developed by serial passage of the wild-type (wt) RVFV ZH548 strain in the presence of mutagen [2]. The MP-12 is a promising live vaccine candidate for both human and veterinary use because the virus is markedly attenuated in mice, nonhuman primates, and ruminants and retains its high immunogenicity [3, 22–27]. However, intraperitoneal (i.p.) inoculation of young mice with MP-12 can result in efficient virus replication in the central nervous system (CNS) (J. Morrill et al, unpublished data). Furthermore, i.p. inoculation of SCID mice with MP-12 results in the development of neurological signs and death of all mice [24]. These data demonstrated that MP-12 can invade and undergoes efficient replication in the CNS of young animals or immunocompromised animals, and implied that the virus potentially replicates in the CNS of humans with immature or impaired immune system. Thus, it is important to develop highly immunogenic RVFV vaccines with reduced or no neurovirulence for mass vaccination programs.

To develop a safe and immunogenic RVF vaccine, we have generated a novel, single-cycle replicable MP-12 (scMP-12), which does not cause systemic infection in immunized hosts [28]. Figure 1a shows our design of the scMP-12 system. scMP-12 carries L RNA; M RNA mutant encoding wt NSm protein, wt

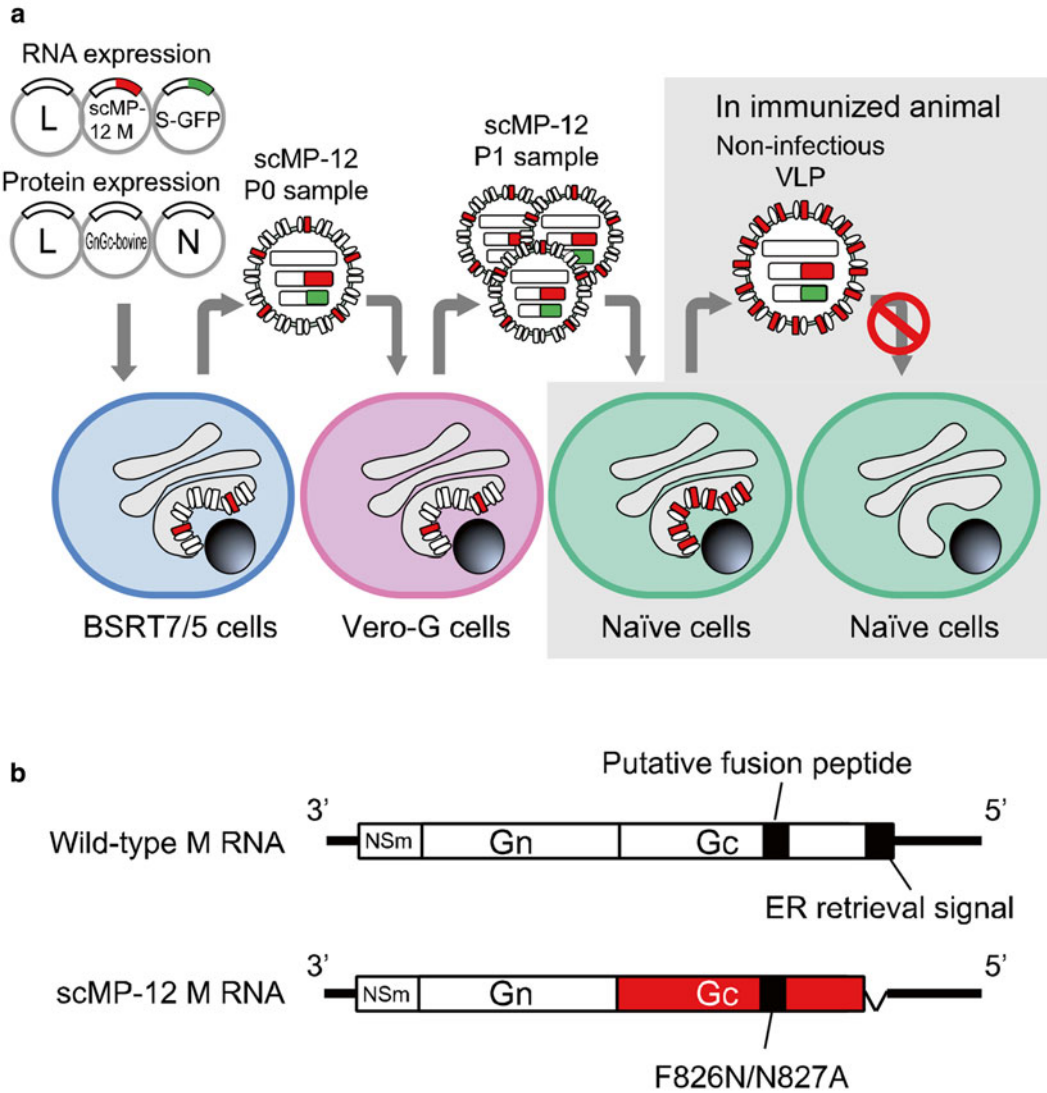


**Fig. 1** Schematic diagram of the MP-12-based scMP-12 system [28]. (**a**) scMP-12 was generated in BSR-T7/5 cells stably expressing T7 polymerase by co-transfection of plasmids, which expressed the L, N, and Gn/Gc proteins, as well as the L RNA, S-GFP RNA, and scMP-12 M RNA encoding Gc mutant. Gn/Gc gene of the protein expression plasmid as bovine codon-optimized sequence. scMP-12 contains viral RNAs and is competent for initiating infection, as it carries wt Gn/Gc derived from the protein expression plasmid. scMP-12 is further propagated in Vero-G cells stably expressing wt Gn/Gc. Inoculation of scMP-12 into naïve cells results in viral RNA synthesis, expression of viral proteins, and production of noninfectious VLPs in immunized animals. (**b**) Schematic diagram of antiviral-sense M RNA and scMP-12 M RNA. The ORFs of NSm and Gn genes are shown in *white boxes*, while the Gc gene ORF appears in the *red box*. The *black bars* represent both the putative fusion peptide and the ER retrieval signal. In scMP-12 M RNA, mutations within the putative fusion peptide and deletion of the ER retrieval signal are shown

78-kDa protein, wt Gn protein, and mutant Gc protein lacking the C-terminal 5-amino-acid-long endoplasmic reticulum retrieval signal and having two amino acid substitution within a putative fusion peptide (Gn/GcΔ5); and S RNA carrying green fluorescent protein (GFP) in place of NSs protein (S-GFP RNA). Due to amino acid substitutions in the putative fusion peptide, Gc protein of scMP-12 is fusion defective. We rescued scMP-12 by using a modified MP-12 reverse genetics system [29], in which BSR-T7/5 cells stably expressing T7 polymerase [30] are co-transfected with three RNA-expression plasmids expressing the L RNA, the mutant M RNA encoding the mutant Gc protein, and S-GFP RNA, as well as three protein expression plasmids encoding the L, N, and Gn/Gc proteins. There is a possibility that the M RNA synthesized from the RNA expression plasmid acquires a wt Gc sequence from the M RNA synthesized from the Gn/Gc protein expression plasmid by homologous RNA recombination, generating infectious MP-12. To reduce a chance of homologous recombination between these two RNA transcripts, the Gn/Gc protein expression plasmid encodes a bovine codon-optimized Gn/Gc sequence. The scMP-12 that is produced from the plasmid-transfected cells is infectious due to the presence of wt Gn/Gc and undergoes amplification in Vero-G cells stably expressing Gn/Gc. In scMP-12-infected naïve cells, intracellular accumulation of all of the viral structural proteins and the production of noninfectious viruslike particles (VLPs) occur. Accordingly, in immunized hosts, scMP-12 undergoes single-cycle replication and produces noninfectious VLPs from infected cells. scMP-12 particles in the inoculum, viral proteins accumulated in scMP-12-infected cells, and released noninfectious VLPs all serve as immunogens to elicit immune responses to RVFV proteins. Due to its characteristic single-cycle replication, the scMP-12 did not cause any sign of neurovirulence after intracranial inoculation into suckling mice. scMP-12-immunized mice elicited neutralizing antibodies and efficiently protected the mice from wild-type RVFV challenge by inhibiting wild-type RVFV replication in various organs and viremia [28].

This chapter describes methods for scMP-12 generation. A flowchart presents an outline of the scMP-12 generation procedure (Fig. 2). We first describe methods for construction of the scMP-12 M RNA expression plasmid and bovine codon-optimized Gn/Gc expression plasmid. Then, procedures are given for scMP-12 generation by using a reverse genetics system. Subsequently, we describe procedures for establishment of Vero-G cells by using a plasmid encoding Gn/Gc proteins and a drug selection maker. Finally, we provide methods for propagation and titration of scMP-12 in Vero-G cells.

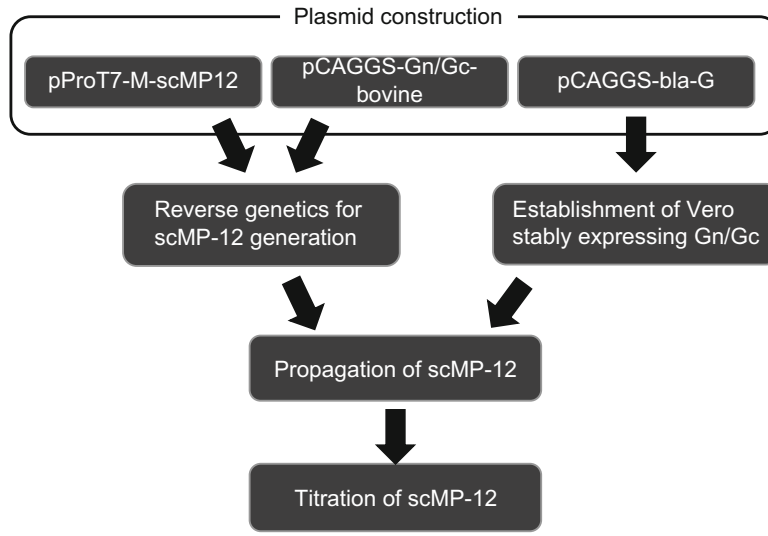


**Fig. 2** A flowchart for generation of scMP-12

## 2 Materials

### *2.1 Plasmid Construction*

1. Proofreading PCR enzyme: Pfu ultra DNA polymerase (Agilent), or equivalent.
2. 10 mM dNTP mix.
3. Agarose, loading dye, and nucleic acid stain suitable for gel electrophoresis.
4. Agarose gel electrophoresis system: 1.0 % agarose gel, use ultrapure agarose (electrophoresis grade) with 1× TBE. Prepare 1 L of 10× TBE stock solution in ultrapure water with 108 g of Tris base, 55 g of boric acid, 40 mL of 0.5 M EDTA (pH 8.0).
5. QIAquick Gel Extraction Kit (Qiagen).
6. QIAquick PCR Purification Kit (Qiagen).
7. 2× Rapid Ligation Buffer (Promega) and T4 DNA ligase (Promega) or equivalent.
8. Chemical-competent cells TOP10 (Invitrogen).
9. SOC medium: Add 20 g tryptone, 5 g yeast extract, 2 mL of 5 M NaCl, 2.5 mL of 1 M KCl, 10 mL of 1 M $MgCl_2$, 10 mL of 1 M $MgSO_4$, 20 mL of 1 M glucose in 1000 mL of $H_2O$. Sterilize by autoclaving and store at room temperature.
10. LB agar plate with ampicillin: Add 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, and 15 g of Bacto Agar (BD Biosciences), in

1000 mL of $H_2O$. Sterilize by autoclaving. After cooling down to 55 °C in a water bath, add 1 mL of 50 mg/mL ampicillin, and mix well. Pour 15 mL of LB/agar media into 10 cm Petri dish. After solidifying the LB/agar, store at 4 °C.

11. 14 mL Round-bottomed, snap-cap tubes, sterile.
12. LB medium with ampicillin: Add 5 g tryptone, 2.5 g yeast extract, and 5 g NaCl in 1000 mL of $H_2O$. Sterilize by autoclaving. After cooling down to room temperature, add 1 mL of 50 mg/mL ampicillin. Store at 4 °C.
13. Qiaprep Miniprep Kit (Qiagen) or equivalent.
14. QIAGEN Plasmid Midi Kit (Qiagen) or equivalent.

#### *2.1.1 Construction of pProT7-M-scMP12 Plasmid*

1. Plasmid: pProT7-M [29].
2. Primer pair: deltaGc-tail-F, AAACGTCTCTTAGATCAGTG CGTGTAAAAGC, and deltaGc-tail-R, AAACGTCTCTTCT AGGCAGCAAGCCAC.
3. Primer pair: M F826N_N827A-F,GAATGTGGAGGATGGG GGTGTGGGTGTAACGCTGTGAACCCATCTT, and M F826N_N827A-R,AAGATGGGTTCACAGCGTTACACCC ACACCCCCATCCTCCACATTG.
4. T7 terminator primer: ATGCTAGTTATTGCTCAGCGG.
5. Restriction enzymes: *Bsm*BI, *Dpn*I (NEB).

#### *2.1.2 Construction of pCAGGS-Gn/Gc-Bovine*

1. Synthetic gene, bovine codon-optimized M RNA expression plasmid, pProT7-M-bovine.
2. BsmEco-MboGnGc-F, AAACGTCTCTAATTCACCATGGC CGGCATCGCCATG, BsmNot-MboGnGc-R, AAAACGTC TCTGGCCGCTCTAGCTAGCTTTTTTTGTAGCAGCC
3. Restriction enzymes: *Eco*RI, *Not*I, *Bsm*BI (NEB).

#### *2.1.3 Construction of pCAGGS-bla-G*

1. Plasmids: pCX4-bsr, and pCAGGS-G.
2. Restriction enzymes: *Not*I, *Stu*I, *Eco*RV (NEB).

### *2.2 Establishment of Vero-G Cells*

1. Restriction enzyme: *Fsp*I.
2. QIAquick PCR Purification Kit (Qiagen).
3. Vero E6 cells.
4. Growth media: DMEM (HyClone) supplemented with 10 % FBS, 1× penicillin/streptomycin (100 unit/mL of penicillin, 100 μg/mL of streptomycin).
5. 6-Well plates.
6. 96-Well plates.
7. Fugene HD transfection reagent (Promega).

8. pCAGGS-Bla-G (Subheading 2.1.3).
9. Opti-MEM (Gibco).
10. Blasticidin S hydrochloride (Gibco).
11. 10 % Formalin in PBS.
12. 0.1 % TritonX-100 in PBS.
13. 2 % BSA in PBS.
14. Anti-Gn monoclonal antibody (R1-4D4) [31].
15. Alexa-594-conjugated anti-mouse IgG (Invitrogen).

### 2.3 scMP-12 Generation by Reverse Genetics

1. BSR T7/5 cells.
2. Glasgow's minimal essential medium supplemented with 10 % FBS, 10 % tryptose phosphate broth, 1 mg/mL G418, 1× penicillin/streptomycin (100 unit/mL of penicillin, 100 μg/mL of streptomycin).
3. 6-Well plate.
4. Plasmids: pProT7-L [29], pProT7-S-GFP [28], pProT7-M-scMP12 (Subheading 2.1.1), pT7IRES-L [29], pT7IRES-N [29], pCAGGS-Gn/Gc-bovine (Subheading 2.1.2).
5. TransIT-LT1 (Mirus).
6. Opti-MEM (Invitrogen).

### 2.4 Propagation of scMP-12 in Vero-G Cells

1. Vero-G cells (Subheading 2.2).
2. Growth media: DMEM 10 % FCS, 1× penicillin/streptomycin (100 unit/mL of penicillin, 100 μg/mL of streptomycin).
3. Supernatant from plasmid-transfected BSR T7/5 cells (Subheading 2.3).
4. 6-Well culture plate.

### 2.5 Plaque Assay for Titration of scMP-12 in Vero-G Cells

1. Vero-G cells (Subheading 2.2).
2. 6-Well culture plate.
3. scMP-12 virus stock solution (Subheading 2.4).
4. 96-Well deep-well plate.
5. Growth media: DMEM 10 % FCS, 1X penicillin/streptomycin (100 unit/mL of penicillin, 100 μg/mL of streptomycin).
6. MEM containing 0.6 % Tragacanth gum (MP Biomedicals), 5 % FBS, and 5 % tryptose phosphate broth.
7. 4 % Paraformaldehyde in PBS.
8. 0.1 % TritonX-100 in PBS.
9. Anti-N rabbit polyclonal antibody, which was generated by injecting a purified, bacterially expressed fusion protein

consisting of glutathione-S-transferase and full-length MP-12 N protein into rabbits.

10. HRP-conjugated anti-rabbit IgG antibody (Santa Cruz).
11. NovaRED peroxidase substrate (Vector Laboratories).

## 3 Methods

### 3.1 Plasmid Constructions

#### 3.1.1 Construction of pProT7-M-scMP12 Plasmid

pProT7-M-scMP12 plasmid is produced from M RNA expression plasmid pProT7-M by deleting five amino acid residues of C-term of Gc protein and introducing F826N/N827A mutation in putative fusion peptide of Gc protein. To generate pProT7-M-scMP12, two steps are required: (1) delete five amino acid residues of C-terminal of Gc first and (2) introduce F826N/N827A mutations.

1. Prepare reaction mixture for PCR amplification,

   40 μL Nuclease-free $H_2O$.
   5 μL 10× reaction buffer.
   1 μL 10 mM dNTPs.
   1 μL Forward primer: deltaGc-tail-F (10 pmol/μL).
   1 μL Reverse primer: deltaGc-tail-R (10 pmol/μL).
   1 μL pProT7-M template at 10 ng/μL concentration.
   1 μL Pfu Ultra DNA Polymerase.

2. Preheat the thermal cycler to 95 °C, then heat the samples at 95 °C for 1 min, and run the thermal cycling program for 30 cycles with the following setting: 30 s at 95 °C, 30 s at 58 °C, and 6 min at 72 °C. Add one cycle at 72 °C for a final extension of 10 min.
3. Run 5 μL of the PCR product by 1 % agarose gel electrophoresis in 1× TBE buffer. Use a 1 kbp DNA ladder to control for the correct product size. Cut out the band, and the extracted PCR fragment by using QIAquick Gel Extraction Kit, eluting fragments with 30 μL of $H_2O$ (*see* **Note 1**).
4. Digest the purified DNA fragment with 1 μL of *Bsm*BI (*see* **Note 2**) and 1 μL of *Dpn*I (*see* **Note 3**) enzymes in reaction volume 25 μL for 1 h at 37 °C and for 1 h at 55 °C. Purify the digested DNA fragments by using a QIAquick PCR purification kit, eluting fragments with 30 μL of $H_2O$.
5. Perform ligation using T4 DNA ligation; add 1 μL of the digested fragments and 5 μL of 2× ligation buffer, 1 μL of T4 DNA ligase, and 3 μL of $H_2O$ into a 1.5 mL tube and incubate for 5 min at room temperature.
6. Transform 50 μL of TOP10 competent cells with the 3 μL of ligated product.

7. Add 100 μL of SOC media to the transformed cells.
8. Incubate with 180–200 rpm shaking at 37 °C for 1 h.
9. Plate onto LB agar plates supplemented with ampicillin for selection and incubate the plates at 37 °C for 16–20 h.
10. Select well-isolated colonies from the transformation plates, inoculate the colonies in 1 mL LB media for bacterial culture with ampicillin, and incubate with 180–200 rpm shaking for 10–16 h at 37 °C.
11. Make plasmid minipreps from the cultures by using the QIAprep Miniprep Kit, eluting fragments with 50 μL of $H_2O$.
12. Determine the DNA concentration in each preparation by measuring the absorbance at 260 nm by using a spectrophotometer.
13. Screen the clones by sequencing using T7 terminator primer.
14. Sequence entire M RNA with clones which possess Gc cytoplasmic tail deletion (*see* **Note 4**). Designate one clone possessing correct sequence as pProT7-Gn/GcΔ5.
15. Perform site-directed mutagenesis by using Quickchange II site-directed mutagenesis kit following the manufacturer's protocol with M F826N_N827A-F and M F826N_N827A-R primer.
16. Add 1 μL of *Dpn*I into PCR reaction tube in **step 15** (*see* **Note 3**).
17. Transform 50 μL of TOP10 competent cells with the 3 μL of ligated product.
18. Add 100 μL of SOC media to the transformed cells.
19. Incubate with 180–200 rpm shaking at 37 °C for 1 h.
20. Plate onto LB agar plates supplemented with ampicillin for selection and incubate the plates at 37 °C for 16–20 h.
21. Select well-isolated colonies from the transformation plates, inoculate the colonies in 1 mL LB broth for bacterial culture with ampicillin, and incubate with 180–200 rpm shaking for 10–16 h at 37 °C.
22. Make plasmid minipreps from the cultures, and using the QIAprep Miniprep Kit, elute fragments with 50 μL of $H_2O$.
23. Determine the DNA concentration in each preparation by measuring the absorbance at 260 nm by using a spectrophotometer.
24. Sequence entire M RNA and choose one clone possessing correct sequence (*see* **Note 4**).
25. Propagate the pProT7-M-scMP12 by midiprep.

### *3.1.2 Construction of pCAGGS-Gn/Gc-Bovine*

1. Synthesize bovine codon-optimized MP-12 Gn/Gc gene (Gn/Gc-bovine) by commercial company.
2. PCR amplify the Gn/Gc-bovine gene to add linker sequence.

*Prepare PCR reaction mix on ice:*

40 μL Nuclease-free $H_2O$.

5 μL 10× Reaction buffer.

1 μL 10 mM dNTPs.

1 μL Forward primer: BsmEco-MboGnGc-F (10pmol/μL).

1 μL Reverse primer: BsmNot-MboGnGc-R (10pmol/μL).

1 μL Template at 10 ng/μL concentration.

1 μL Pfu Ultra DNA polymerase.

3. Preheat the thermal cycler to 95 °C, then heat the samples at 95 °C for 1 min, and then run the thermal cycling program for 30 cycles with the following setting: 30 s at 95 °C, 30 s at 58 °C, and 6 min at 72 °C. Add one cycle at 72 °C for a final extension of 10 min.
4. Analyze 5 μL of PCR product on a 1 % agarose gel with ethidium bromide in 1× TBE-buffer. Use a 1 kbp DNA ladder to control for the correct product size.
5. Purify the PCR product using QIAquick PCR purification kit, eluting fragments with 30 μL of $H_2O$.
6. Digest the purified DNA fragment with 1 μL of *Bsm*BI enzyme in 40 μL reaction volume for 1 h at 55 °C (*see* **Note 5**). Simultaneously, digest 1 μg of pCAGGS-G with 1 μL of *Eco*RI and 1 μL of *Not*I enzymes in 25 μL reaction volume for 1 h at 37 °C.
7. Run the digested PCR product and plasmid by 1 % agarose gel electrophoresis with ethidium bromide in 1× TBE buffer, cut out the band, and extract DNA fragment by using the QIAquick Gel Extraction Kit, eluting fragments with 30 μL of $H_2O$, respectively.
8. Perform ligation reaction using T4 DNA ligation; add 1 μL of the digested PCR fragment and 1 μL of the digested vector and 5 μL of 2× ligation buffer, 1 μL of T4 DNA ligase, and 2 μL of $H_2O$ into 1.5 mL tube and incubate for 5 min at room temperature.
9. Transform 50 μL of TOP10-competent cells with the 3 μL of ligated product.
10. Add 100 μL of SOC media to the transformed cells.
11. Plate onto LB agar plates supplemented with ampicillin for selection and incubate the plates at 37 °C for 16–20 h.
12. Select well-isolated colonies from the transformation plates, inoculate the colonies in 1 mL LB media for bacterial culture with ampicillin, and incubate with 200 rpm shaking for 10–16 h at 37 °C.

13. Make plasmid minipreps from the cultures, using the QIAprep Miniprep Kit, eluting fragments with 50 μL of $H_2O$.
14. Determine the DNA concentration in each preparation by measuring the absorbance at 260 nm by using a spectrophotometer.
15. Sequence the either open reading frame of Gn/Gc (*see* **Note 4**).
16. Propagate pCAGGS-Gn/Gc-bovine with midiprep.

### *3.1.3 Construction of pCAGGS-bla-G*

1. Digest pCX4-bsr with 1 μL of *Eco*RV and 1 μL of *Not*I enzymes in 25 μL reaction volume for 1 h at 37 °C. Simultaneously, digest 1 μg of pCAGGS-G with 1 μL of *Not*I and 1 μL of *Stu*I enzymes in 25 μL reaction volume for 1 h at 37 °C.
2. Run the digested pCX4-bsr and pCAGGS-G by 1 % agarose gel electrophoresis, respectively. Extract pCX4-bsr *Not*I-*Eco*RV fragment (1254 bp) and pCAGGS-G *Not*I-*Stu*I fragment (7471 bp) using the QIAquick Gel Extraction kit, eluting fragments with 30 μL of $H_2O$.
3. Perform ligation reaction using T4 DNA ligation; add 1 μL of the pCX4-bsr *Not*I-*Eco*RV fragment and 1 μL of the digested vector and 5 μL of 2× ligation buffer, 1 μL of T4 DNA ligase, and 2 μL of $H_2O$ into 1.5 mL tube and incubate for 5 min at RT.
4. Transform 50 μL of TOP10-competent cells with the 3 μL of ligated product.
5. Add 100 μL of SOC media to the transformed cells.
6. Plate onto LB agar plates supplemented with ampicillin for selection and incubate the plates at 37 °C for 16–20 h.
7. Select well-isolated colonies from the transformation plates, inoculate the colonies in 1 mL LB broth for bacterial culture with ampicillin, and incubate with 200 rpm shaking for 10–16 h at 37 °C.
8. Make plasmid minipreps from the cultures, by using the QIAprep Miniprep Kit, eluting fragments with 50 μL of $H_2O$.
9. Identify clones with correct insert size by restriction digestion.
10. Determine the DNA concentration in each preparation by measuring the absorbance at 260 nm using a spectrophotometer.
11. Sequence the entire insertion region.
12. Propagate pCAGGS-bla-G (Fig. 3) with plasmid midiprep kit.

## *3.2 Generation of Vero-G Cells*

To obtain a Vero cell line stably expressing Gn and Gc proteins, Vero cells were transfected with a plasmid encoding Gn/Gc and a drug selection gene. We screened individual cell clones for Gn

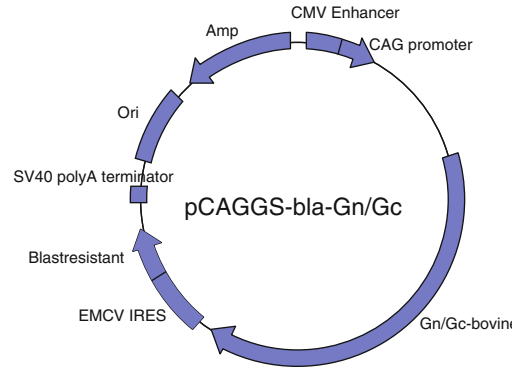


**Fig. 3** Map of pCAGGS-bla-Gn/Gc. RVFV Gn/Gc and blasticidin-resistant genes were flaked with encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) sequence

expression levels by using indirect immunofluorescence with an anti-Gn monoclonal antibody (R1-4D4), isolated a cell clone expressing highest levels of Gn, and designated it as Vero-G cells.

1. To linearize pCAGGS-bla-G, digest 10 μg of pCAGGS-bla-G with 2 μL of *Fsp*I enzyme in 100 μL reaction volume for 1 h at 37 °C (*see* **Note 6**).
2. Purify the digested pCAGGS-bla-G product by using QIAquick PCR purification kit, eluting fragments with 30 μL of $H_2O$.
3. Determine the DNA concentration in each preparation by measuring the absorbance at 260 nm using a spectrophotometer.
4. Seed $3 \times 10^5$ cells/well of Vero E6 cells at least two wells of a 6-well plate in DMEM medium supplemented with 10 % FBS and incubate the plates in a 37 °C cell incubator overnight. The cell density should be approximately 80 % confluency at the time of transfection.
5. Add 200 μL OPTI-MEM (Invitrogen) and 3 μg of pCAGGS-bla-G to a 1.5 mL tube. Add 9 μL of FuGENE HD (Promega) and mix by vortexing. Incubate for 15 min at room temperature. The entire transfection mixture was added to a well dropwise. Add 200 μL of OPTI-MEM to at least one well of the plate as mock transfection.
6. Incubate the plate overnight at 37 °C, 5 % $CO_2$.
7. Aspirate the media from the cells and add DMEM medium containing 10 % FCS and 20 μg/mL of blasticidin. Incubate the plate for 5–7 days at 37 °C, in a 5 % $CO_2$ atmosphere until all mock-transfected cells die (*see* **Note 7**).
8. Clone the cells by limiting dilution. Wash the cells once with PBS, add 0.2 mL of EDTA-trypsin, and incubate for 5 min at

37 °C. Confirm that all cells are detached. Add 2 mL of DMEM medium containing 10 % FCS and 20 μg/mL of blasticidin and suspend the cells. Adjust cell concentrations to 0.5–1 cells/well in medium containing 20 μg/mL of blasticidin, and dispense a 100 μL cell suspension to each well of 96-well culture plates.

9. Incubate these plates at 37 °C in a 5 % $CO_2$ atmosphere until cells are visible to the naked eye (*see* **Note 8**). Screen wells forming single colony.
10. Trypsinize the cells with 50 μL of trypsin-EDTA and resuspend in 200 μL of media. Divide into two and seed into two 48-well plate. Incubate overnight at 37 °C in a 5 % $CO_2$ atmosphere.
11. Wash the cells with 200 μL of PBS once.
12. Fix with 100 μL of 10 % formalin for 10 min at room temperature.
13. Remove formalin and wash the cells three times with 200 μL of PBS.
14. Add 100 μL of 0.1 % TritonX-100 in PBS and incubate for 10 min at room temperature for permeabilization.
15. Remove TritonX-100 in PBS and wash the cells three times with 200 μL of PBS.
16. Add 200 μL of blocking reagent (2 % BSA in PBS) and incubate for 60 min at room temperature.
17. Remove blocking reagent.
18. Add 100 μL of anti-Gn monoclonal antibody (R1-4D4) to the wells in PBS and incubate for 30 min at room temperature (*see* **Note 9**).
19. Remove antibody containing PBS and wash three times with 200 μL of PBS.
20. Add 100 μL of Alexa594-conjugated anti-mouse IgG to the wells in PBS and incubate for 30 min at room temperature.
21. Remove antibody containing PBS and wash three times with 200 μL of PBS.
22. Observe under fluorescent microscopy and select a cell clone expressing highest levels of Gn and designate as Vero-G cells.
23. Propagate the selected clone cells in TC75 flask and make cell stocks.

### 3.3 scMP-12 Generation by Reverse Genetics

1. Seed ~$5 \times 10^5$ BSR T7/5 cells/well of a 6-well plate (*see* **Note 10**).
2. Incubate cells for 24 h in growth medium. Cells should be 60–80 % confluent at the time of transfection.

3. Premix DNAs for transfection:
   (a) Use 1.1 μg each of the pProT7-L, pProT7-M-scMP12, and pProT7-S-GFP RNA expression plasmids and pT7IRES-N protein expression plasmids.
   (b) Use 0.55 μg of pCAGGS-Gn/Gc-bovine and pT7IRES-L protein expression plasmids.
   (c) This gives a total of 5.5 μg of DNA to be transfected.
4. Transfection:
   (a) Add 200 μL of OPTI-MEM and premixed DNAs into a 1.5 mL tube.
   (b) Add 2 μL transfection reagent (TransIT-LT1, Mirus) per μg of DNA (*see* **Note 11**).
   (c) Incubate transfection mixture for 15 min at room temperature.
5. Aspirate media from wells and add 2 mL of growth medium, and, then, add transfection mixture dropwise to cells.
6. Incubate the plate for 10 days at 37 °C, 5 % $CO_2$. Check GFP-positive cell spreading under fluorescent microscopy every day (*see* **Note 12**).
7. Collect the virus-containing supernatant.
8. Remove cell debris by centrifugation (10,000 × *g*, for 5 min, at 4 °C).
9. Aliquot and store at −80 °C.

### 3.4 Propagation of scMP-12 in Vero-G Cells

1. Seed $5 \times 10^5$ Vero-G cells/well of a 6-well plate in growth medium (DMEM 10 % FCS, 1× penicillin/streptomycin).
2. Incubate cells for 24 h at 37 °C and 5 % $CO_2$; cells should be near confluent at the time of infection.
3. Remove media.
4. Add 300 μL of undiluted virus-containing supernatant to Vero-G cells (*see* **Note 13**).
5. Infect Vero-G cells for 1 h at 37 °C and 5 % $CO_2$.
6. Wash Vero-G cells once with growth medium to remove virus inoculum.
7. Incubate cells with growth medium for 4–5 days at 37 °C and 5 % $CO_2$.
8. Observe cells daily to confirm GFP signal spreading under fluorescence microscopy: GFP signal can be typically observed within 18 h of infection.
9. When ~90 % of Vero-G cells become GFP positive, harvest virus-containing supernatant.

10. Remove cell debris by centrifugation (10,000 × *g*, for 5 min, at 4 °C).
11. Transfer virus-containing supernatant to fresh tube.
12. Aliquots the virus-containing supernatant and store at −80 °C. One aliquot should be used for determination of the virus titer described below.

### 3.5 Plaque Assay

1. Seed $5 \times 10^5$ Vero-G cells/well of a 6-well plate in growth medium.
2. Incubate cells for 24 h at 37 °C and 5 % $CO_2$; cells should be near confluent at the time of infection.
3. Prepare a tenfold serial dilution of the virus stock solution (from $10^{-1}$ to $10^{-5}$ dilution): add 450 μL of media into each well of a 96-well deep-well plate. Add 50 μL of the virus stock solution to be tested into the first well and carefully pipette up and down. Then transfer 50 μL of this first dilution into the second well and continue as described for the first dilution to generate a serial dilution.
4. Remove media from 6-well-plated Vero-G cells.
5. Add 400 μL of virus dilutions prepared in Vero-G cells. Include negative control by providing cells with medium lacking virus inoculum.
6. Incubate the 6-well plates for 1 h at 37 °C and 5 % $CO_2$. Rock the plate gently back and forth every 15 min to keep the cells covered by virus diluent.
7. Aspirate the inoculum.
8. Wash the Vero-G cells once with media.
9. Add 2 mL of MEM containing 0.6 % Tragacanth gum (MP Biomedicals), 5 % FBS, and 5 % tryptose phosphate broth to each well and incubate the plates for 4 days at 37 °C and 5 % $CO_2$.
10. On day 4, remove the Tragacanth gum-containing media from 6-well plates.
11. Wash the cells with 400 μL of PBS once.
12. Fix with 400 μL of 4 % paraformaldehyde for 10 min at room temperature.
13. Remove paraformaldehyde solution and wash the cells three times with 1 mL of PBS.
14. Add 400 μL of 0.1 % TritonX-100 in PBS and incubate for 10 min at room temperature.
15. Remove TritonX-100 in PBS and wash the cells three times with 1 mL of PBS.

16. Add 400 μL of blocking reagent (2 % BSA in PBS) and incubate for 60 min at room temperature.
17. Remove blocking reagent.
18. Add 400 μL of anti-N rabbit polyclonal antibody to the wells in PBS and incubate for 30 min at room temperature (*see* **Note 14**). Remove antibody containing PBS and wash three times with 1 mL of PBS.
19. Add 400 μL of HRP-conjugated anti-rabbit IgG to the wells in PBS and incubate for 30 min at room temperature. Remove antibody containing PBS and wash three times with 1 mL of PBS.
20. The plaques were visualized with Nova RED peroxidase substrate (*see* **Note 15**) (Fig. 4).
21. Remove Nova RED peroxidase substrate solution.
22. Count plaques in wells where the plaques can clearly be distinguished and calculate the plaque-forming units (pfu). Count plaques in each well and determine the PFU per milliliter using the following formula: PFU/mL = number of plaques × dilution factor × 2.5.

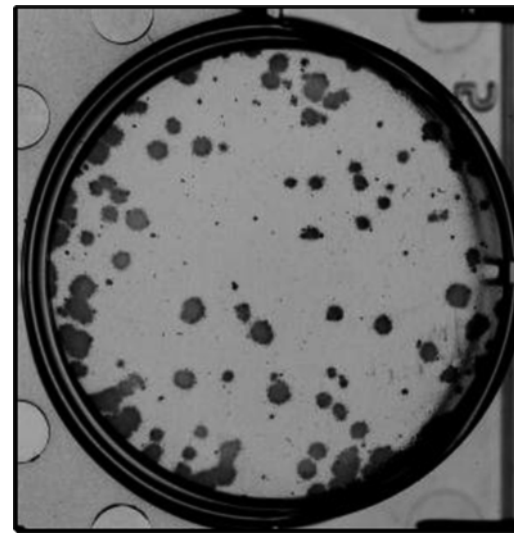

**Fig. 4** Plaque formation of scMP-12 in Vero-G cells. Vero-G cells were infected with scMP-12 and overlaid with medium containing Tragacanth gum. After the cells were fixed, plaques were stained with anti-N antibody and visualized by using a Nova RED peroxidase substrate

## 4 Notes

1. Illumination of short-wavelength UV (254 nm) to DNA causes pyrimidine dimers, resulting in low transformation efficiency. To avoid this, long-wave UV (365 nm) or blue LED is useful.
2. Cleavage site of *Bsm*BI is downstream of the recognition site. Cleavage product does not contain the recognition site, allowing desired plasmid construction introducing insertion, deletion, and mutations (Fig. 5).
3. *Dpn*I digests only methylated plasmid produced in *E. coli* and does not digest non-methylated DNA, such as PCR products. Therefore, *Dpn*I digestion of PCR products only removes template plasmids.
4. Design sequencing primers for entire MP-12 M, which is available in Genbank (Accession number is DQ380208). Typically,

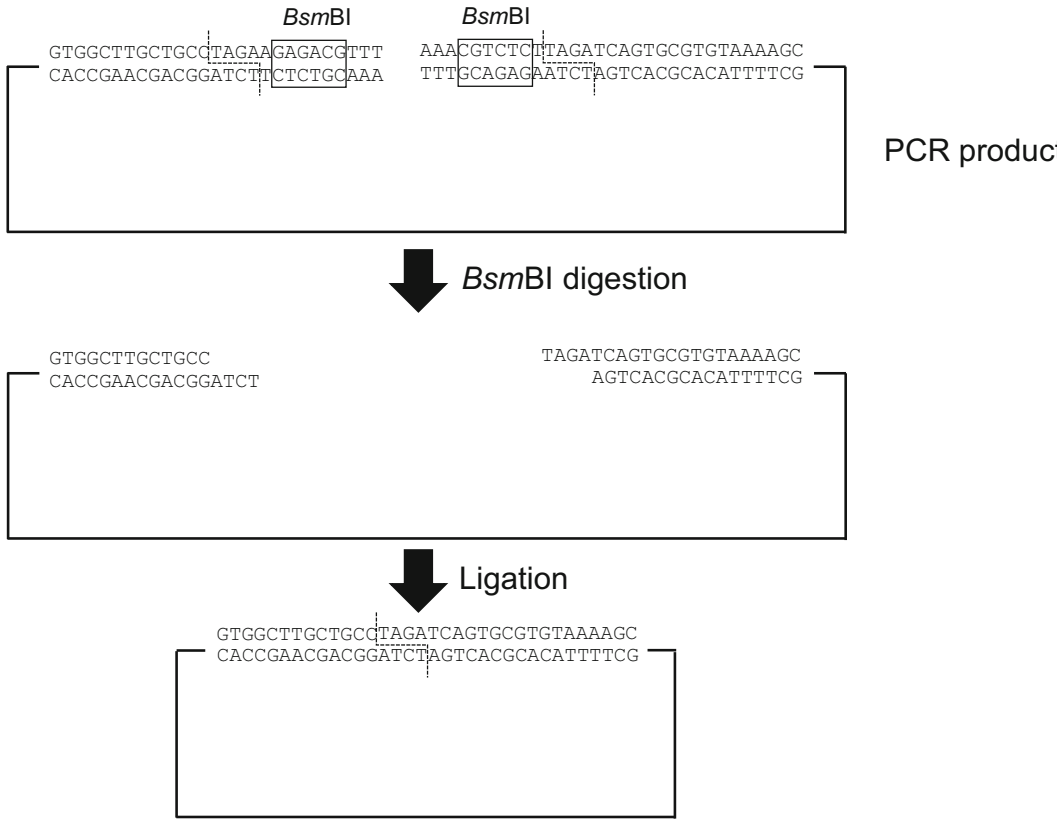


**Fig. 5** Cloning strategy for the construction of pProT7-Gn/GcΔ5. (1) PCR product contains *Bsm*BI recognition sites. (2) Incubation of the PCR products with *Bsm*BI generates the same 5′- and 3′-terminal nucleotide overhangs, which can be self-ligated. (3) Cloning product deleted nucleotides encoding 5 amino acid residues from C-terminal end of Fc. *Black boxes*: *Bsm*BI recognition sites

in designing primers a 600–700 nt interval works for the regular sequencer. It depends on the performance of a sequencer.

5. Bovine codon-optimized M sequence contains *Eco*RI site. *Bsm*BI digests downstream of the recognition site with four-nucleotide overhangs. Forward and reverse primers are designed to possess four-nucleotide overhangs of *Eco*RI and *Not*I digestion by *Bsm*BI digestion, respectively.
6. Linearization of plasmid enhances the integration efficiency into cell genomes.
7. When transfected cells become confluent during drug selection, passage the cells once with 1:10 dilution.
8. Typically, 10–18 days are required for forming a visible plaque.
9. If anti-Gn antibody is not available, anti-RVFV polyclonal antibody may work for this screening.
10. If BSR T7/5 cells are not available, BHK cells stably expressing T7 RNA polymerase may work for generation of scMP-12.
11. Selection of transfection reagent is important for this experiment. TranIT-LT1 is one of the most suitable reagents for reverse genetics for RVFV production.
12. The viral titer of scMP-12 will reach its plateau when most of the cells turn to GFP positive.
13. If viral titers are too high, nearly 100 % of Vero-G cells become GFP positive at 1 day postinfection. In this case, a 10–100 times dilution is required to avoid production of defective interfering particles.
14. Anti-RVFV polyclonal antibody does not work in this experiment since anti-RVFV antibodies pick up Gn/Gc signals, causing high background.
15. If the signals are weak, an avidin-biotin complex (ABC) staining method may help to enhance the signals.

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