Chapter 1

Oncolytic Herpes Simplex Virus Engineering and Preparation

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Abstract

Herpes simplex virus-1 (HSV-1) is an enveloped, double-stranded DNA virus that has been used with modification as an oncolytic virus against a number of tumor types. Modifications that make HSV-1 replication-conditional, i.e., selectively divide in replicating cells make it fulfill a prerequisite criteria for oncolytic viruses. Other appealing features of HSV-1 as an oncolytic virus include its large, modifiable genome; its sensitivity to antiviral agents, such as ganciclovir; and its lack of host cell integration. Here, we review the methods of HSV-1 engineering, through traditional recombination techniques as well as through bacterial artificial chromosome (BAC) technology. We then describe protocols for titering, amplification, and purification of engineered HSV-1-derived oncolytic viruses.

Key words: Oncolytic virus, Virus titer, Virus purification, HSV-BAC, G47Δ

1. Introduction

Herpes simplex virus-1 (HSV-1) is an enveloped, double-stranded DNA virus with several advantages for use as an oncolytic virus: a large genome suitable for insertion of foreign genes; tropism for neural cells; a safety mechanism in its sensitivity to agents, such as ganciclovir; high titers can be generated; and it does not integrate into the host genome, so it is unlikely to be oncogenic (1, 2). These advantages have led to the use of HSV-1s genetically engineered to be replication-conditional, i.e., selectively dividing in replicating cells, as oncolytic viruses in the treatment of numerous cancer types (2–4). Here, we describe the basic structure of wild-type HSV-1 and the protocols for modification, purification, amplification, and titration of HSV-1 for use as an oncolytic virus. We also describe several specific examples of oncolytic HSVs modified with the methods presented here.
HSV-1 has four main components: a core that contains double-stranded DNA (dsDNA), an icosadeltahedral capsid, an amorphous tegument, and an outer lipid bilayer envelope with glycoprotein spikes \((3, 4)\). The DNA is arranged as two unique sequences – Unique Long \((U_L)\) and Unique Short \((U_S)\) – that are each flanked by repeat sequences. Homologous recombination at these repeat sequences results in four possible permutations of linear and inverted \(U_L\) and \(U_S\) sequences. During infection and after cell entry mediated by surface glycoproteins, the viral genome circularizes and begins transcription of immediate early (IE) genes, which are involved with transcription regulation of the host cell and virus. The IE genes also contribute to transcription regulation of early (E) and late (L) genes \((2–4)\).

The first engineered HSV-1 oncolytic virus had a mutation in the viral thymidine kinase (TK) gene, and showed killing of glioma cells in vitro and in models of glioma in vivo \((5, 6)\). This mutant is replication-conditional because the mutant virus can only replicate in dividing cells because only dividing cells like tumor cells express sufficient amounts of mammalian TK to complement the lack of viral TK. Unfortunately, viral expression of TK is essential for the antiviral efficacy of nucleoside analogues like ganciclovir or acyclovir. Viral thymidine kinase monophosphorylates these nucleoside analogues (viral TK is much more efficient than human nucleoside kinases at monophosphorylating antiviral nucleoside analogues), which are then further phosphorylated by cellular kinases, ultimately producing ganciclovir or acyclovir triphosphate, which are incorporated into elongating DNA chains, after which they interrupt DNA synthesis. Because of the inability to use nucleoside analogues as a safety mechanism for this first-generation, viral-TK mutated vector, significant safety concerns were raised and, in fact, neurotoxicity was seen at high doses \((6)\).

In an alternative HSV-1 vector, a mutation in the viral \(\gamma_{34.5}\) gene was introduced. The \(\gamma_{34.5}\) gene and its product, ICP34.5, allow normal HSV to subvert the host’s “shut-off” response against infection. Once infected with HSV, a normal cell will activate protein kinase R (PKR) which in turn phosphorylates and inactivates eukaryotic initiation factor-2\(\alpha\) (eIF-2\(\alpha\)), thereby shutting down protein synthesis in the normal host cell. ICP34.5 restores protein synthesis by activating protein phosphatase-1\(\alpha\) which dephosphorylates and restores eIF-2\(\alpha\) function \((2, 6)\). Mutations in this gene, \(\gamma_{34.5}\), result in an HSV that cannot replicate in normal cells, which abrogate protein synthesis machinery. In malignant cells, however, the activation of PKR is less pronounced, likely due to other mutations, and thus, the ICP34.5-mutant HSV is replication-conditional. Another viral gene that can be mutated to render HSV-1 replication-conditional is \(U_L 39\), which encodes ICP6, the large subunit of viral ribonucleotide reductase. HSVs with ICP6 mutations can only replicate in dividing cells like tumor cells because only tumor cells
express sufficient levels of mammalian ribonucleotide reductase to complement the viral mutation. However, while ICP34.5 and ICP6 single mutant HSVs are sensitive to antiviral nucleoside analogues and lack that specific safety concern seen with the original TK-deficient HSVs, ICP34.5 and ICP6 single mutant HSVs have undergone few preclinical studies because of the concern that single mutant HSVs would pose a safety risk if they were to undergo in vivo recombination and restoration of the wild-type HSV phenotype (2).

Double-mutant viruses are theoretically safer as the chances of recombination and restoration of the wild-type HSV phenotype are decreased (2, 6, 7). Therefore, a second-generation virus, termed G207, was created which harbors an insertion of the Escherichia coli lacZ gene into the U1,39 gene, which encodes ICP6, and has deletion of both copies of ICP34.5 (7). MGH-1, which has the same modifications as G207, was created by a different group using a slightly different transfection method (8).

Finally, a third generation HSV-1 mutant, G47Δ, was created by modifying G207 by using bacterial artificial chromosome (BAC) technology to delete the nonessential viral gene α47 (9, 10). The deletion created in G47Δ places the late US11 gene under control of the immediate-early α47 promoter, which enhances the growth of G47Δ relative to G207 because earlier expression of US11 prevents the premature termination of protein synthesis that slows the growth of γ34.5-mutated HSVs like G207. In addition, deletion of the α47 gene prevents the downregulation of MHC class I expression seen in HSVs expressing α47, which could enhance the antitumor immune response (11). Importantly, G47Δ was produced using BAC technology, an advance which allows for more straightforward HSV-1 engineering, including the “arming” of oncolytic HSVs to express other gene products that might improve oncolytic virotherapy.

Here, we describe the initial preparation and storage of wild type and recombinant HSV-1, as can be obtained either commercially or through another laboratory. Next, we review the recombination methods used in engineering oncolytic HSVs. We also review protocols for oncolytic HSV production and titration.

## 2. Materials

### 2.1. Materials Needed for HSV Work of Any Kind

1. Biosafety level 2 (BL2) and a biocontainment hood: HSV-1 is a human pathogen and requires these precautions.

2. A virucidal agent such as LpHse (Steris): 7.7% o-phenylphenol, 7.6% p-tertiary amylphenol, 84.7% inert ingredients. Used at a 1:256 dilution (1/2 oz/gal distilled water).
1. African green monkey kidney cells [Vero cells, American Type Culture Collection (ATCC)].
2. DMEM tissue culture medium (Mediatech, Inc.).
3. Fetal bovine serum (FBS) and calf serum (CS) (Mediatech, Inc.).
4. Heat-inactivated FBS (iFBS): Made by heating FBS to 56°C for 30 min and then stored at −20°C.
5. 1% Penicillin/streptomycin solution (Invitrogen).
6. Sterile Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 50 mg/L streptomycin sulfate, 100 mg/L kanamycin monosulfate, 1,000 mg/L glucose, 36 mg/L sodium pyruvate, 0.133 g/L calcium chloride (CaCl₂·2H₂O), and 0.1 g/L magnesium chloride (MgCl₂·6H₂O) (Sigma-Aldrich).
7. Sterile PBS (Mediatech, Inc., Manassas, VA) enriched with 1,000 mg/L glucose.
8. BD Falcon 6-well tissue culture plates and T150 tissue culture flasks (BD Biosciences).
9. Human anti-HSV IgG (Gamunex, Talecris Biotherapeutics).
10. Ethanol–dry ice bath (Sigma-Aldrich).
11. Sonicator bath at 4°C, standard tabletop tissue culture centrifuge, standard tabletop rocking shaker, standard tissue culture microscope, and high-speed floor centrifuge (Fisher Scientific, Inc.).
12. Virus buffer: 150 mM NaCl and 20 mM Tris–HCl at pH = 7.5 (Sigma-Aldrich).
13. Virus stock (e.g., Strain F obtained commercially or virus stock obtained from previous amplifications/purifications as outlined below).
14. X-Gal concentrated stock solution: 50 mg/mL X-Gal substrate (Sigma-Aldrich) dissolved in DMSO. Solution should be stored at 4°C in the dark (aluminum foil wrap around a 50-mL conical tube).
15. Diluted X-Gal solution that can be used once the day of titration: 0.5 mL of X-Gal stock (50 mg/mL), 0.05 mL of 1 M MgCl₂, 1 mL of 125 mM potassium ferricyanide (in PBS), and 1 mL of 125 mM potassium ferrocyanide (in PBS) mixed in PBS for a total volume of 25 mL (PBS in the final solution should come out to 1×). Filter X-Gal solution after mixing to prevent crystal formation.
16. Neutral red solution: 7 mL of ddH₂O, 1 mL of methanol, and 40 μL of neutral red stock solution (Sigma-Aldrich).
17. Glutaraldehyde (0.2%)/paraformaldehyde (2%) solution (Sigma-Aldrich).
2.3. Materials for Engineering Oncolytic HSV
(Subheadings 3.5–3.12)

1. HSV-G47Δ BAC DNA: Obtained as described by Fukuhara et al. (10). HSV-G47Δ BAC expresses GFP, chloramphenicol resistance, an FRT locus, and a loxP locus within the ICP6 gene.

2. pVec9 shuttle vector: Obtained as described by Fukuhara et al. (10), contains a loxP site, a lacZ gene, a transgene multiple cloning site with a pCMV promoter, an FRT site, and a kanamycin resistance gene.

3. 10× Cre buffer: 500 mM Tris–HCl, pH 7.5, 330 mM NaCl, 100 mM MgCl₂.

4. TE buffer: 10 mM Tris–HCl and 1 mM EDTA (pH = 8.0) (Sigma-Aldrich).

5. 50× TAE buffer, pH 7.6–7.8: 242 g Tris–HCl, 100 mL 0.5 M EDTA, pH 8.0, and 57.1-mL glacial acetic acid; then, add enough volume of Millipore H₂O to dissolve solids, then add HCl to get final pH to 7.6–7.8, then use Millipore H₂O to get final volume to 1,000 mL.

6. 3 M Sodium acetate, glucose, ethanol, glycerol, isopropanol, agarose, TAE buffer (Sigma-Aldrich).

7. LB plates with kanamycin (10 µg/mL) and chloramphenicol (12.5 µg/mL) (Sigma-Aldrich).

8. Shaking incubator at 37°C (Fisher Scientific, Inc.).

9. SOC medium (per protocol by QIAGEN).

10. Electroporation cuvettes with 0.1-cm gap (Gene Pulser, Bio-Rad, Inc.).

11. E. coli electroporation competent cells DH10B (Invitrogen).

12. Screwtop microcentrifuge tubes (Fisher Scientific, Inc.).

13. Gene Pulser Xcell electroporator (Bio-Rad, Inc.).

14. Solution 1 (25 mM Tris–HCl, 10 mM EDTA, 50 mM glucose): Add 250 µL of 1 M Tris–HCl (pH 8.0), 200 µL of 0.5 M EDTA, and 90 mg of glucose into a total of 10 mL of ddH₂O (Sigma-Aldrich).

15. Lysozyme 25 mg/mL: Add 250 mg of lysozyme (Sigma-Aldrich) to 10 mL of 10 mM Tris–HCl at pH 8.0.
16. QIAGEN plasmid mini and maxi kits, including buffers QBT, QC, and QF (QIAGEN).
17. QIAGEN-tip 500 columns (QIAGEN, Dusseldorf).
18. DNA spectrophotometer (Fisher Scientific, Inc.).
19. Low- and high-molecular weight DNA ladders (Invitrogen).
20. Standard DNA electrophoresis setup, including loading dye (Bio-Rad, Inc.).
21. Sarstedt centrifuge tubes (Nümbrecht).
22. pFLPe plasmid (available from laboratory of Prof. Rudolf Jaenisch, MIT, Cambridge, MA).
23. Opti-MEM solution without any supplements (Invitrogen).
25. RSB buffer: 10 mM Tris–HCl at pH 8.0, 10 mM KCl, and 1.5 mM MgCl$_2$ (Sigma-Aldrich).
26. Phenol, chloroform, and isoamyl (purification grade) (Sigma-Aldrich).

3. Methods

Subheadings 3.1–3.4 below deal primarily with the titration, amplification, and purification of HSV-1, protocols which can be applied not only to wild-type HSV-1, but also to modified virus. In short, when any HSV-1 virus is received from an outside source, such as a vendor or another laboratory, it needs to be quantified in order to know the correct amounts for amplification. Once amplified in a cell line, the virus should be purified and requantified to serve as a stable virus stock.

Subheadings 3.5–3.12 are protocols to make an “armed” oncolytic virus from the G47Δ backbone (10). These protocols can also be applied in general to manipulation and purification of oncolytic viruses. For example, the transfection procedure to transfect plasmid DNA into a cell line can be used for simple recombination to modify standard backbones. A procedure similar to the one presented here for G47Δ manipulation was used for the creation of MGH-1, which was created from R3616, a virus from the strain F backbone that has both copies of ICP34.5 deleted (12) (see Note 1).

3.1. Virus Titration

1. Establish Vero cell culture in 6-well tissue culture plates day before titration at approximately $3 \times 10^5$ cells/2 mL of DMEM supplemented with 10% CS and 1% penicillin/streptomycin.
2. Grow cells overnight.
3. On the day of titration, ensure that cells are approximately 60–70% confluent and appear healthy.
4. Wash twice with virus wash buffer (DPBS with supplements and 1% iFBS) and ensure that cells are not dry during steps.

5. While cells are in last wash, prepare virus stock by performing three freeze–thaw cycles followed by sonication at 30% output for 1 min.

6. Use a serial dilution technique to create $10^{-2}$ and $10^{-4}$ dilutions of virus stock in virus wash buffer (e.g., 10 μL into 1 mL of virus wash buffer for $10^{-2}$. Then, take 10 μL of new solution and place into 1 mL of virus wash buffer for $10^{-4}$ dilution of original virus).

7. Add 0.7 mL of each virus dilution per well (three wells per dilution).

8. Rock 6-well plate at room temperature for 5 min.

9. Place 6-well plate at 37°C for 1–2 h.

10. Remove virus and add 2 mL/well with DMEM supplemented with 1% iFBS and 0.1% human IgG at 37°C.

11. Incubate infected cells appropriately for approximately 2–3 days until plaques develop. Plates should be level in incubator.

12. If titrating virus with lacZ gene insertion, proceed to protocol 1.1B, otherwise, remove media and fix with methanol (2 mL/well) for 5 min.

13. Remove methanol and let cells dry.

14. To stain and count cells, incubate each well with 1 mL of Giemsa stain that is prediluted 1:20 in ddH₂O. Ensure that stain is evenly distributed.

15. Incubate in stain for 5–10 min carefully checking intensity of stain.

16. Once appropriately stained, remove Giemsa stain and rinse with tap water gently.

17. Dry plates and count plaques using stereomicroscope to obtain virus titer, namely plaque-forming units (pfu) per amount of total virus stock used.

18. Repeat with various dilutions as necessary to obtain plates that can easily be counted for pfu. This will allow for the most accurate titer to be obtained.

1. Follow protocol in Subheading 3.1 until cells are ready for staining.

2. Wash cells with sterile PBS without calcium/magnesium. Note: if cells do not adhere well to the plate, do not wash before fixation.

3. Remove wash carefully.
4. Warm X-Gal staining solution to 37°C fully and mix well before use.

5. Fix cells with cold glutaraldehyde/paraformaldehyde fixative (see Subheading 2) at 2 mL/well and let sit for 5–10 min at room temperature.

6. Wash cells three times with sterile PBS without calcium/magnesium.

7. Incubate with X-Gal staining solution (see Subheading 2) at 0.6 mL/well at 37°C for 3 h in nonsterile incubator.

8. Aspirate remaining X-Gal stain and wash cells with tap water at room temperature.

9. Counterstain with neutral red solution (see Subheading 2) for 2–10 min at room temperature while noting color change. When color has changed appropriately, quench with tap water and count plaques as described above (Fig. 1).

10. Plates can be stored in the open at room temperature.

### 3.3. Virus Amplification in Vero Cells

1. Grow healthy low-passage Vero cells in two large T150 tissue culture flasks on the day before amplification at $8 \times 10^6$ cells/flask.

2. The next day, cells should be approximately ten million cells per T150 or 70–80% confluent. Ensure that they appear healthy.

3. Aspirate media and wash cells twice with PBS and 1% iFBS at 37°C.
4. Dilute virus stock in PBS and 1% iFBS to obtain an MOI of 0.01 just prior to infection. MOI is obtained from initial titration above.

5. Place approximately 8 mL of diluted virus into each T150 flask of Vero cells at 37°C.

6. Rock flasks slowly for 10 min at room temperature.

7. Incubate at 37°C and 5% CO₂ for 90 min. Ensure that flask remains level horizontally during infection.

8. Aspirate viral inoculum and add 25 mL of DMEM with 3% iFBS per 150-mL flask and incubate at 34.5°C and 5% CO₂.

9. Wait until complete cytopathic effect has been obtained when cells are rounded and refractile, but have not yet detached from the plate. This is the optimal time for harvesting of virus with the highest titers.

10. When total CPE has been obtained, dislodge cells by firmly banging flasks on hard surface and by scraping cells if necessary.

11. Pipette cells gently up and down on flask surface to mix cells and assist in removing cells from flask surface.

12. Centrifuge cells in tabletop tissue culture centrifuge at approximately 2,500 × \( g \) for 5 min.

13. Remove all but approximately 1 mL of supernatant.

14. Add 1 mL of virus buffer (for a total volume of 2 mL) and resuspend pellet in cryotubes.

15. Rapidly freeze in EtOH–dry ice bath and store at −80°C.

16. Repeat procedure until a total of ten flasks are prepared and stored to provide a total 20 mL of amplified, but unpurified virus stock in a 1:1 mix of media and virus buffer.

**3.4. Virus Purification (see Note 2)**

1. Combine 20 mL of amplified virus stock from Subheading 3.1 into a 50-mL BD Falcon centrifuge tube.

2. Freeze/thaw 3× in EtOH–dry ice bath.

3. Sonicate at 30% output for 1 min at 4°C.

4. Spin tubes at 500 × \( g \) for 10 min in standard tissue culture tabletop centrifuge.

5. Remove supernatant and respin supernatant at 2,500 × \( g \) for 10 min.

6. Save last supernatant and filter using 5 \( \mu \)m Millex SV (PVDF) filter followed by 0.45-\( \mu \)m Millex-HV (durapore/PVDF) filter. Use approximately 10 mL per filter. Since virus tends to stick to tubes, use a little virus buffer to rinse tubes and filter.

7. Filter 2 mL of sterile 30% sucrose in PBS with 0.22-\( \mu \)m Millex PVDF filter and add to bottom of presterilized 10-mL Oak Ridge centrifuge tubes.
8. Layer 5 mL of double-filtered virus supernatant from step 5 very carefully over sucrose–PBS cushion in Oak Ridge tubes. This is done by adding the supernatant very slowly over the side of the tube, so two layers are clearly visible.

9. Centrifuge at 22,000 × g for 90 min at 4°C in floor-top high-speed centrifuge.

10. Remove supernatant and resuspend pellet carefully in 2 mL of PBS with 10% glycerol. PBS with 10% glycerol should be pre-filtered with 0.22 μm PVDF filter.

11. Let pellet stand in PBS + 10% glycerol at 4°C for 2 h on shaker.

12. After 2 h, sonicate on high for 30 s, vortex, and pipette up and down to create homogenous solution of virus.

13. Aliquot small portion for titering of virus and place remainder in cryotubes for storage at −80°C.

The goal of this section is to produce a large DNA known as BAC-G47/Vec9/transgene, which has both chloramphenicol and kanamycin resistance as well as GFP and lacZ expression.

1. If needed, specific transgenes can be inserted into the pVec9 shuttle vector through standard recombination protocols, generating pVec9/transgene.

2. Combine 1 μg of HSV-G47Δ BAC DNA, 1 μL 10× Cre buffer, 1 μL Cre recombinase, and approximately 50 ng of pVec9 shuttle vector into a total volume of 10 μL of RNAse/DNAse-free sterile H₂O.

3. Incubate mixture for 30 min at 37°C.

4. Inactivate recombinase by incubating for 10 min at 70°C.

5. Incubate at room temperature for 10 min.

6. To begin ethanol precipitation, add 40 μL of TE buffer.

7. Add 5 μL of 3 M sodium acetate and mix gently by pipetting.

8. Add 125 μL of ethanol and invert very gently. Since the DNA is large, aggressive mixing will damage it.

9. Incubate at −80°C for 30 min.

10. Centrifuge in tabletop microcentrifuge for 15 min at 4°C at 16,500 × g.

11. Discard supernatant and wash pellet with 500 μL of 70% ethanol.

12. Centrifuge in tabletop microcentrifuge for 5 min at 4°C at 16,500 × g.

13. Discard the supernatant and dry pellet to air, but do not let it dry out completely.

14. Dissolve in 5 μL of sterile H₂O.
After the initial recombination in Subheading 3.5 has occurred, BAC-G47Δ/Vec9/transgene is formed. In this section, we electroporate BAC-G47Δ/Vec9/transgene into bacteria, which can then be used to select for appropriate recombination events through double-antibiotic resistance genes.

1. Chill four electroporation cuvettes on ice.
2. Warm two antibiotic-selection plates at 37°C.
3. Thaw on ice one frozen vial of DH10B E. coli cells.
4. Transfer 20 μL of E. coli cells each to four new screwtop microcentrifuge tubes cooled on ice.
5. Immediately before each transformation add 1 μL of DNA mixture obtained from Subheading 2.1. Mix completely by tapping gently.
6. Transfer cell/DNA mixture to chilled electroporation cuvette on ice and tap it gently so that the mixture sits evenly. There should be no air bubbles and any condensation on the outside of the cuvette should be wiped carefully.
7. Use the following electroporator settings: 1.5 kV, 200 Ω, 25 μF.
8. Wipe off any condensation on the side of cuvette and place it in the chamber.
9. Remove the cuvette immediately from the chamber and add 500 μL of SOC medium at room temperature.
10. Transfer the E. coli diluted in SOC medium to sterile microcentrifuge tube.
11. Incubate in shaking incubator at 37°C for 45 min.
12. Plate 100–400 μL of the cells into two antibiotic-selective plates and incubate overnight at 37°C. Exact volume of cells used should be titrated to efficiency of transduction.

1. From the two antibiotic-selective plates from Subheading 2.2, pick individual colonies and inoculate each clone into a separately labeled microcentrifuge tube with 10 μL of sterile ddH2O.
2. Create a replica plate by taking 1 μL of the picked colonies from step 1 and plate it on another antibiotic plate that is demarcated in grid fashion. Grow overnight and then store plate at 4°C.
3. Take remaining 9 μL of bacteria in ddH2O and amplify in 10 mL of LB plus antibiotics. Grow overnight in standard shaking conditions. Create stock by making five labeled cryotubes and adding 850 μL of bacteria in LB and 150 μL of sterile glycerol per tube. Snap-freeze in EtOH–dry ice bath and store at −80°C. This should leave approximately 5 mL of amplified bacteria in LB for DNA isolation.
4. Harvest bacterial cells by centrifugation at $9,000 \times g$ in standard tabletop centrifuge for 10 min at 4°C. Discard supernatant. (After centrifugation, it is possible to store cells at −20°C for later work).

5. Resuspend cell pellet in 200 μL of Solution 1 without lysozyme.

6. Transfer to new microcentrifuge tube.

7. Add 25 μL lysozyme (see Subheading 2) and mix gently.

8. Add 400 μL of P2 from QIAGEN kit and mix well gently. Leave on ice for 2 min.

9. Add 400 μL of P3 from QIAGEN kit and mix well gently. Keep on ice for 5 min.

10. Centrifuge for 10 min at 13,000 rpm in standard tabletop microcentrifuge at 4°C.

11. Transfer supernatant to a new 2-mL microcentrifuge tube. The total volume should be approximately 1 mL at this stage.

12. Add 1 μL of RNAse from QIAGEN kit at 100 mg/mL to make a final concentration of 0.1 mg/mL.

13. Incubate at room temperature for 15 min.

14. Precipitate DNA by adding 700 μL of room temperature isopropanol.

15. Centrifuge immediately for 10 min at 16,500 × $g$ (microcentrifuge) at room temperature.

16. Discard supernatant and wash pellet with 500 μL of 70% ethanol at room temperature.

17. Centrifuge for 5 min at 16,500 × $g$ (microcentrifuge) at room temperature.

18. Remove the supernatant and allow pellet to air-dry for 1 min at 37°C.

19. Dissolve pellet with 50 μL of TE buffer.

20. Measure DNA concentration using spectrophotometer at optical density of 260 nm per standard procedures.

### 3.8. Hind III Restriction Analysis and DNA Gel Electrophoresis

1. Combine 20 μL of 10× NEBuffer 2 and 2 μL of Hind III enzyme together with 78 μL of ddH$_2$O for a total of 100 μL of digestion mix.

2. Take five clones of BAC-G47Δ/Vec9/transgene from Subheading 3.5 and combine 10 μL of each sample clone with 10 μL of digestion mix. Incubate at 37°C for 2 h. Ideally, there should be at least 1 μg of DNA for the digestion mix to work properly.

3. BAC-G47Δ and BAC-G47Δ/Vec9/empty should be used as controls. For each control sample, combine 1 μL of DNA,
9 µL of ddH₂O, and 10 µL of digestion mix. Incubate at 37°C for 2 h.

4. For gel electrophoresis, create a 0.5% agarose/TAE 300-mL gel using standard procedures.

5. Load 20 µL of each sample and 2 µL of loading dye into each lane.

6. Two DNA ladders should be used – a low-molecular weight and a high-molecular weight ladder. For low-molecular weight ladder, follow manufacturer’s instructions. To make high-molecular weight ladder, combine 4 µL of high-molecular weight DNA ladder with 14 µL of TE. Incubate in water bath at 65°C for 5 min. Add 2 µL of loading dye and load total 20 µL into lane.

7. Run gel at 50 V for 16–24 h.

8. Once the correct clones are identified, amplify these clones and appropriate controls by inoculating clones from the replica plate into 2 mL of LB/chloramphenicol (12.5 µg/mL) and incubating in shaking incubator at 37°C for 3 h.

9. Add this 2 mL preculture to a flask of 200 mL LB/chloramphenicol (12.5 µg/mL) and grow overnight with good aeration in shaking incubator at 37°C.

10. Centrifuge 200 mL of bacterial culture at 2,500 × g for 10 min at 4°C. Remove supernatant by decantation.

11. Resuspend cell pellet in 16 mL of Solution 1 (without lysozyme).

12. Add 1.6 mL of lysozyme (see Subheading 2) and mix gently.

13. Add 32 mL of P2 and mix well gently. Leave at room temperature for 5 min.

14. Add 32 mL of P3 and mix well gently. Keep on ice for 10 min.

15. Centrifuge for 30 min at 5,000 × g in standard floor centrifuge at 4°C.

16. Transfer supernatant to a new centrifuge tube.

17. Centrifuge again for 30 min at 5,000 × g in standard floor centrifuge at 4°C.

18. Transfer the supernatant to a clean Erlenmeyer flask through a layer of sterile gauze.

19. Add 80 µL of RNase at 100 mg/mL to make a final concentration of 0.1 mg/mL.

20. Incubate at room temperature for 15–30 min.

21. Using a QIAGEN-tip 500 column, preequilibrate columns with 10 mL of QBT buffer.
15. Apply DNA solution to the column (approximately 2.5× of 30-mL volume).
16. Warm 15 mL of QF buffer to 65°C.
17. Wash columns with 30 mL of QC three times.
18. Elute with 15 mL of QF buffer at 65°C into a Sarstedt 30-mL tube.
19. Precipitate DNA by adding 10.5 mL of room temperature isopropanol to each tube and mix gently.
20. Centrifuge for 60 min at 8,000×g in floor-top centrifuge at 4°C.
21. Resuspend the DNA pellet with 15 mL of 80% ethanol.
22. Centrifuge for 15 min at 8,000×g in floor-top centrifuge at 4°C.
23. Remove the supernatant and resuspend the pellet in 1 mL of 70% ethanol. Transfer to new screwcap microcentrifuge tube.
24. Centrifuge at 16,500×g in tabletop microcentrifuge for 10 min at 4°C.
25. Remove supernatant.
26. Pulse centrifuge to remove any residual ethanol.
27. Dry the pellet for 2 min at room temperature without letting it overdry.
28. Dissolve the pellet with 100 μL of TE buffer.
29. Dilute 4 μL of DNA into a total volume of 200 μL of MQ. Measure optical density at 260 nm and calculate concentration of DNA.
30. Repeat Hind III digestion and gel electrophoresis after “maxi-prep” amplification to ensure that the appropriate DNA has been obtained for transfection. This DNA is referred to as BAC-G47Δ/Vec9/transgene.

3.10 Transfection of G47Δ/Vec9/Transgene and pFLPe into Vero Cells

Cotransfecting the BAC-G47Δ/Vec9/transgene plasmid along with pFLPe, a plasmid that expresses FLPe, into Vero cells ensures that, within the Vero cell, expression of FLPe will create another recombination event, generating G47Δ-transgene that expresses lacZ but not GFP. Transcription of this DNA produces viral particles for further purification and amplification. Of note, the large BAC-G47Δ/Vec9/transgene does not produce viral particles without the FLPe recombination due to the presence of a lambda stuffer sequence.

1. Establish Vero cell culture in 6-well tissue culture plates day before titration at approximately 3×10^5 cells/well with 2 mL of DMEM supplemented with 10% CS.
2. Grow cells overnight.
3. On the day of transfection, ensure that cells are approximately 70–80% confluent and appear healthy.

4. For each well, prepare one tube with 1.5 μg of pHSV-BAC-shuttle, 0.5 μg of pFLPe, and 250 μL of Opti-MEM I without any supplements. Gently mix.

5. For each well, prepare another tube with 20 μL of lipofectamine 2,000 and 250 μL of DMEM. Gently mix and incubate for 5 min at room temperature.

6. Combine tubes from steps 5 and 6. Gently mix and incubate for 20 min at room temperature.

7. While incubating the combined mixture from both tubes, remove media from 6-well plate and wash with DMEM once.

8. Add 2 mL of DMEM + 1% iFBS to each well after the wash.

9. Add combined DNA-lipofectamine mix to each well and rock gently for 5 min at room temperature.

10. Incubate at 37°C for 4 h.

11. Remove media and replace with DMEM + 1% iFBS. Ensure that there are GFP-positive cells when changing the media.

12. When 50–80% CPE is observed, harvest viral particles and cells by scraping.

13. Centrifuge cells in tabletop tissue culture centrifuge at approximately 700 × g for 5 min.

14. Remove all but approximately 1 mL of supernatant.

15. Add 1 mL of virus buffer (for a total volume of 2 mL) and resuspend pellet in cryotubes.

16. Rapidly freeze in EtOH–dry ice bath and store at −80°C.

17. In order to plaque-purify by limiting dilution in the next step, perform titering of virus stock as described in Subheading 3.1. Since this new virus has the recombination with the *LacZ* insertion, the X-Gal staining protocol in Subheading 3.2 should be followed.

Plaque-purification by limiting dilution will isolate a single viral clone that can be further tested to ensure accuracy of the recombination.

1. On the day before the titration, plate Vero cells in 96-well plates at 1 × 10^4 cells/well with 150 μL of DMEM supplemented with 10% CS. Make enough plates to be able to detect positive recombinant viruses at <0.5 pfu/well.

2. Remove media using a multichannel pipetter and wash cells with DPBS supplemented with glucose and 1% iFBS.

3. Dilute recombinant virus in DPBS supplemented with glucose and 1% iFBS at a concentration that will be sufficient to infect cells at 0.3 pfu/well.
4. Remove media from cells and infect cells at 0.3 pfu/well in 50 μL. Rock for 10 min at room temperature and incubate at 37°C for 2 h on a rocker.

5. After 2 h, remove virus inoculum. Ensure that inoculum is treated with bleach and disposed properly.

6. Add 150 μL DMEM and 1% iFBS (without human IgG) and incubate at 37°C.

7. On day 2, check the wells for plaques under the microscope and mark wells that contain only one plaque.

8. When plaques are large enough (usually on day 4), scrape cells and transfer both cells and media to a new labeled 96-well plate using a multichannel pipetter. Seal the plate with parafilm and store at −35°C in a box clearly labeled as biohazard.

9. Pick a few clones out of the marked wells and titer virus using X-gal staining as outlined in Subheading 3.2.

Once titer is determined, the viral DNA needs to be amplified and purified to confirm that the correct recombination has taken place.

1. On day 0, plate Vero cells in 1T150 flask per clone using DMEM supplemented with 10% CS. Cells should be approximately 90–95% confluent on day 1. It is important to have control clones as well.

2. Remove media and wash cells with DPBS supplemented with glucose and 1% iFBS.

3. Dilute virus at MOI 1.5 in 2 mL of PBS supplemented with glucose and 1% iFBS. Add to flask.

4. Rock flask for 10 min and incubate at 34.5°C for 2 h with rocking every 15 min.

5. Add 13 mL of DMEM supplemented with 1% iFBS and incubate at 34.5°C until complete cytopathic effect (approximately 24 h).

6. On day 2, remove media (If there are many cells in the media, spin cells at 700 × g for 5 min and resuspend in 1 mL of PBS. This can be added to cells in the next step). Dislodge cells by gentle tapping of flask. Scrape off attached cells that remain. Transfer into 15-mL BD Falcon centrifuge tube.

7. Wash any remaining cells in flask with 2 mL of PBS and add to centrifuge tube with other cells. At the end of this, there should be one centrifuge tube with as many cells as possible from one flask.

8. Spin cells at 500 × g for 5 min in standard tabletop centrifuge. Discard supernatant and wash cells with 10 mL RSB. Spin again at same settings and resuspend with 1 mL RSB.
9. Let cells sit on ice for 20 min to allow swelling. For the following steps, ensure that cells and supernatants are kept at 4°C until the proteinase K is added.

10. Add 50 µL of 10% NP40 to give final concentration of 0.5% in 1 mL of RSB and cells. Incubate at room temperature for 10 min.

11. Add 10 µL of RNAse A (10 mg/mL in ddH2O) and mix gently.

12. Spin tube at 700 × g for 5 min in standard tabletop centrifuge. Remove the cytoplasmic supernatant and save at 4°C.

13. Resuspend the pellet, which contains the nuclei, in 0.4 mL RSB. Add 10 µL of 10% Triton X-100. Invert gently and spin down at 2,000 rpm for 5 min in standard tabletop microcentrifuge.

14. Combine both cytoplasmic and nuclei supernatants.

15. Add 70 µL of 0.2 M EDTA and 60 µL of 10% SDS to the combined supernatants. Mix gently to avoid air bubbles.

16. Add 25 µL of proteinase K (10 mg/mL in 2 mM CaCl2 and 50 mM Tris–HCl at pH = 8.0).

17. Incubate overnight at 37°C.

18. On day 3, note total volume of sample. Extract DNA by adding a half volume of phenol and another half volume of chloroform–isoamyl alcohol extraction mixture (phenol–chloroform–isoamyl should be at the following ratio – 25:24:1).

19. Rotate gently and centrifuge at room temperature for 5 min at 3,000 × g (microcentrifuge). After phases are separated, remove and save aqueous phase into an Oak Ridge centrifuge tube using a wide bore or cut pipette tip.

20. Repeat extraction on nonaqueous phase using only a half volume of chloroform–isoamyl alcohol (24:1). Save aqueous phase in previous tube.

21. Repeat extraction on nonaqueous phase using only half volume of chloroform (without isoamyl alcohol) and let it evaporate under chemical hood for 15 min.

22. Combine all aqueous phases and note new volume.

23. Begin precipitation of DNA by adding sodium acetate (3 M stock) so that final concentration is 0.3 M. Mix gently.

24. Next, add 2.5 times the volume of ethanol.

25. Allow mixture to precipitate for 2 h at −20°C.

26. Centrifuge in high-speed floor-top centrifuge at 4°C for 30 min at 7,000 × g.

27. Add 1 times the volume of ethanol without disrupting the pellet. Respin at 100 × g for 5 min at 4°C.
28. Let the pellet dry to air briefly and resuspend in 50 μL of TE buffer. Transfer to microcentrifuge tube and wash original tube with another 25–50 μL of TE buffer and add to previous DNA.

29. Measure DNA using spectrophotometer and perform restriction enzyme digestion followed by gel electrophoresis to confirm proper virus. Once confirmed, this virus may be amplified and purified by the methods outlined in Subheading 1.

4. Notes

1. All institutional safety guidelines should be followed for the safe handling and decontamination/disposal of materials that come into contact with the virus, which usually includes disinfecting with virucidal agent then autoclaving cell culture materials that have come into contact with HSV-1 particles, including but not limited to pipettes, flasks, culture media, and tubes. In tissue culture, HSV-1 infected plates need not be separated from uninfected plates because virus transmission only occurs through fluids during sonification by the generation of an aerosol.

2. In Subheading 3.4, if the homogenous solution of virus remains turbid, repeat steps 1–13.

3. Throughout Subheadings 3.5–3.12, appropriate controls should be made and processed as well, including BAC-G47\Delta and BAC-G47\Delta/Vec9/empty. BAC-G47\Delta is the HSV BAC DNA without the Cre-mediated recombination and BAC-G47\Delta/Vec9/empty is the BAC DNA after recombination with an empty shuttle vector that contains no transgene.

4. In Subheading 3.6, once a few colonies have started to grow overnight, it is important to isolate clones and check for accuracy of recombination before creation of the virus.

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