Rescue, Purification, and Characterization of a Recombinant HSV Expressing a Transgenic Protein

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Abstract

In the previous chapter, we describe the engineering of a HSV-BAC genome by *galK* recombineering. Here we describe the procedures to reconstitute, or regenerate, the replicating recombinant virus, and the methods to purify it and characterize it for the correct expression of the transgene. We present the example of R-115, a recombinant expressing murine interleukin 12 (mIL12) from the US1–US2 intergenic region. A specific method for the production of highly purified virions by iodixanol gradient, suitable for in vivo applications, is also detailed.

Key words HSV rescue, Plaque purification, Plaque assay, Virion purification, mIL12 transgene expression

1 Introduction

In the previous chapter we describe the engineering by *galK* recombineering in *E. coli* of an HSV-BAC carrying a transgenic cassette, exemplified by the HSV-BAC 115 recombinant, engineered to encode murine IL12 (mIL12). Here we describe the rescue of the recombinant virus R-115 [1] in susceptible and permissive mammalian cells, the production of purified virions suitable for animal experimentations, and the evaluation of the transgenic mIL12 expression.

2 Materials

2.1 Cells, Cell Culture, and Transfection

1. SK-OV-3 cells were purchased from ATCC and were grown in RPMI Medium 1640-GlutaMAX-I with 100 units/mL penicillin and 100 μg/mL streptomycin (hereafter "RPMI-

GlutaMAX"), supplemented with 10% heat-inactivated FBS (hereafter "FBSΔ").

2. HSV-1 BAC DNA (e.g., R-115; [1]).
3. Phosphate buffered saline (PBS): 9.0 g NaCl, 795 mg Na_2H-$PO_4{\cdot}H_2O$, 144 mg KH_2PO_4 in 800 mL ddH_2O, adjust pH to 7.4, make up to 1 L with ddH_2O, sterilize, store at 4 °C.
4. Trypsin–EDTA (0.05%) in PBS without calcium and magnesium.
5. 2× DMEM.
6. 5% agarose stock solution: dissolve 5 g of cell culture grade Sea Plaque Agarose (Lonza) in 100 mL ddH_2O, autoclave for 20 min at 121 °C. While still hot, mix well. Store at room temperature.
7. Agarose overlay (1%): Melt the 5% agarose stock solution in a microwave oven, let it cool at 45 °C in water bath; in the meantime prewarm 2× DMEM and RPMI-GlutaMAX at 45 °C. For 100 mL final volume of agarose overlay (1% final concentration), mix 20 mL of 5% agarose, 20 mL of 2× DMEM, 54 mL of RPMI-GlutaMAX, and 6 mL FBSΔ (6% final concentration). Keep at 45 °C until ready for use.
8. Lipofectamine 2000 (Life Technologies).
9. Acidic wash solution: 40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3; autoclave.
10. 70% ethanol.
11. 0.67% methyl cellulose (4000 centipoise) in RPMI-GlutaMAX/6% FBSΔ.

2.2 Iodixanol solutions

1. Solution A. OptiPrep Sigma [60% (w/v) solution of iodixanol in water, sterile].
2. Solution B. Diluent (200 mL): mix 5.8 mL of 5 M NaCl (0.85% final), 60 mL of 200 mM HEPES (60 mM final), adjust pH to 7.4 with 1 M NaOH, make up to a final volume of 200 mL with ddH_2O. Sterilize through a 0.2 μm filter.
3. Solution C: 50% iodixanol WS (working solution, for cushion): mix 5 volumes of OptiPrep (Solution A) with 1 volume of Diluent (Solution B).
4. Solution D: HEPES buffered saline (200 mL): 5.8 mL 5 M NaCl (0.85% final), 10 mL 200 mM HEPES (10 mM final), adjust pH to 7.4 with NaOH 1 M, make up to a final volume of 200 mL with ddH_2O. Sterilize through a 0.2 μm filter.
5. Solution E: 25% iodixanol: mix equal volumes of 50% iodixanol (Solution C) and HEPES buffered saline (Solution D).

2.3 Determination of Viral Genome Copies by qPCR

1. DNaseI.
2. Resuspension buffer: 10 mM Tris–HCl, 75 mM NaCl, 1 mM $MgCl_2$, 0.02% PS-80, 5% sucrose, 0.1 mM EDTA, 10 mM L-histidine, 0.5% ethanol, pH 7.4.
3. 0.5 M EDTA (pH 8.0): 186.1 g $Na_2EDTA{\cdot}2H_2O$ in 800 mL ddH_2O, adjust pH to 8.0 with NaOH, make up to 1 L with ddH_2O, aliquot and sterilize by autoclaving.
4. 0.2% SDS in ddH_2O.
5. Proteinase K, 20 μg/mL in ddH_2O.

2.4 Detection of Transgene Expression by ELISA, FACS, or Western Blot

1. Liquid nitrogen.
2. Fixing solution (e.g., methanol, 4% paraformaldehyde, ethanol).
3. 0.1%/1% Triton X-100 in ddH_2O.
4. Diluent for antibodies (immunostaining): 20% FBS in PBS.
5. AP buffer: 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.6.
6. Substrate for alkaline phosphatase: 330 μg/mL NBT (nitro-blue tetrazolium chloride) and 165 μg/mL BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) in AP buffer.
7. FACS buffer: 2% FBS in PBS.
8. Lysis butter: 20 mM HEPES pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% IGEPAL, 0.3 mM Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride, 0.3 mM Nα-p-tosyl-L-phenylalanine chloromethyl ketone.

2.5 Antibodies

1. Primary antibodies: MAb R1.302 (gift from Dr. Marc Lopez, INSERM Marseille, or purchased from Santa Cruz Biotechnology) to nectin1; MAb 9G6 (Santa Cruz) to HER2; PAb R140 to HVEM (gift from Dr. Gary Cohen, University of Pennsylvania); MAb 52S to HSV gH [2].
2. Secondary antibodies: anti-mouse and anti-rabbit Alexa Flour 488-conjugated antibodies.

2.6 Kits

1. Genomic DNA extraction kit.
2. Mouse IL-12 p70 ELISA kit (Thermo Scientific or R&D).
3. ECL Western blotting detection kit.
4. Reverse transcription kit.

2.7 Equipment

1. Bench instrumentation: benchtop centrifuges, microplate reader, Western Blot apparatus and immunodetection system, ultrasound sonicator, spectrophotometer, water bath, rocking platform.

2. Microscopes: fluorescence microscope, inverted microscope, stereomicroscope.
3. Molecular biology: PCR and RealTime-PCR thermal cyclers, gel electrophoresis system, gel imaging system.
4. CO_2 incubator for mammalian cell cultures.
5. Ultracentrifuge equipped with swing out (e.g., Beckman SW28), fixed angle (e.g., Beckman Type 45 Ti), and vertical (e.g., Beckman VTi 65) rotors and suitable tubes, Tube Sealer.
6. T25 and T175 tissue culture flasks.
7. 6-, 12-, and 24-well tissue culture plates.
8. 1.5 and 2 mL Eppendorf tubes.
9. 50 mL conical tubes.
10. Pasteur pipettes.
11. Cell scrapers.
12. 0.8 μm cellulose nitrate membrane filters.
13. Nitrocellulose or PVDF membranes.

3 Methods

3.1 Rescue of Recombinant Virus (R-115) from BAC-DNA by Transfection

The following protocol refers to transfection of SK-OV-3 cells. Transfect at least four recombinant clones, in duplicate.

1. The day before transfection, seed 4×10^5 SK-OV-3 cells per well of a 12-well tissue culture plate in RPMI-GlutaMAX/10% FBSΔ. Incubate overnight at 37 °C in a CO_2 incubator and allow cells to become 60–70% confluent.
2. Next day, dilute 4 μL of Lipofectamine 2000 in 100 μL of medium without antibiotics and serum, mix gently and incubate at room temperature for 5 min.
3. Meanwhile, dilute 0.5–1 μg of quantified HSV-BAC DNA in 100 μL of medium without antibiotics and serum (*see* **Note 1**).
4. Mix gently together the diluted DNA and Lipofectamine 2000, and incubate at room temperature for 20 min.
5. In the meantime, wash cell monolayers once with medium without antibiotics and serum, remove the medium and add 1.3 mL of RPMI-GlutaMAX/2.5% FBSΔ with antibiotics (*see* **Note 2**).
6. After the incubation time, gently transfer drop-by-drop the DNA–Lipofectamine mix on cells using a pipette with wide-orifice tip.
7. Incubate cells for 2–3 days at 37 °C in a CO_2 incubator. No medium replacement is needed following transfection.

8. Allow plaques to develop for 2–3 days. Check the monolayers under the fluorescence microscope for the expression of EGFP reporter. In case you find many plaques in a well, detach cells with a scraper and transfer with the medium in a 2 mL Eppendorf tube. Sonicate or freeze at −80 °C to release intracellular recombinant virus and make the Seed (the first virus culture to be used to start amplified virus cultures). Sonication is set at 12 μm peak to peak. Keep frozen at −80 °C for long-term storage.
9. Seed 1×10^6 SK-OV-3 cells per well of a 6-well tissue culture plate in RPMI-GlutaMAX/10% FBSΔ. Incubate overnight at 37 °C in a CO_2 incubator.
10. Thaw the Seed lysate at 37 °C in a water bath and infect the SK-OV-3 monolayer in the 6-well tissue culture plate from **step 9** with 1 mL of the Seed lysate per well. Place the plate on a rocking platform at 37 °C for 1.5 h.
11. Remove inoculum, add RPMI-GlutaMAX/2.5% FBSΔ and incubate for 2–3 days. This recombinant virus at passage 1 (p1) will serve for further analysis (e.g., transgene sequencing) and plaque purification (*see* Subheading 3.2).

3.2 Plaque Purification

1. Seed 5×10^5 SK-OV-3 cells per well of a 12-well tissue culture plate in RPMI-GlutaMAX/10% FBSΔ. Incubate overnight at 37 °C in a CO_2 incubator and allow cells to become confluent.
2. Infect monolayers with 350 μL of tenfold dilutions of recombinant virus from passage 1 (p1). Place the plate on rocking platform at 37 °C for 1.5 h. After virus adsorption, replace the viral inoculum with agarose overlay (*see* **Note 3**). Incubate at 37 °C in a CO_2 incubator for 3–5 days and monitor the formation of plaques.
3. The day before plaque picking, seed SK-OV-3 cells in 12-well tissue culture plates as in **step 1**. In wells containing only a few plaques, mark well-separated plaques under the stereomicroscope or fluorescence microscope (*see* **Note 4**).
4. Pick at least four single plaques by pushing a sterile glass Pasteur pipette through the agarose overlay. Transfer the agarose plugs by pipetting up and down several times in 500 μL medium in a sterile 1.5 mL Eppendorf tube. Vortex and disrupt the agarose plug by 15 s sonication.
5. Infect SK-OV-3 cells with 350 μL of the undiluted plaque medium and 350 μL of three tenfold dilutions, from 10^{-1} to 10^{-3}. Place the plate on rocking platform at 37 °C for 1.5 h. Store the rest of the undiluted plaque medium at −80 °C.
6. Carry out two additional rounds of plaque purification (**steps 3–5**).

7. After the third round of plaque purification, infect monolayers of SK-OV-3 seeded in 6-well tissue culture plates or T25 flasks (~10^5 cells/cm^2) to amplify the four plaque-purified recombinant viruses. Incubate for 2 days.
8. Choose the well or flask that contains the higher number of plaques, detach cells by trypsinization and reseed all of them in the same well or flask (1:1 trypsinization). Incubate for 2–3 days, or until infection is complete.
9. Detach the cells with a cell scraper and freeze the sample at −80 °C to lyse the cells and release the intracellular recombinant virus. Titer the lysate (*see* Subheading 3.5).
10. Extract the DNA from 200 to 300 μL of infected cell lysate of the selected plaque-purified recombinants by using a Genomic DNA Extraction kit. Confirm the presence of the transgene (e.g., mIL12) by molecular assays.

3.3 Concentration of Extracellular Virions by Ultracentrifugation

The virion purification protocol entails a preliminary centrifugation in order to concentrate extracellular virions from the infected cell medium, with near 100% recovery (*see* **Note 5**). Additional downstream purification steps may be included to decrease the amount of contaminating cellular DNA and proteins.

1. Seed 11 T175 flasks with 1.8 × 10^7 SK-OV-3 cells in 25 mL RPMI-GlutaMAX/10% FBSΔ per flask and incubate overnight at 37 °C in a CO_2 incubator (*see* **Note 6**). Allow cells to become 80–100% confluent (it is not recommended to let cells become over confluent).
2. Trypsinize one T175 flask and determine cell number. Proceed if it is in the range of 1.8–2 × 10^7 cells/T175.
3. Infect the 10 T175 flasks at MOI 0.5 PFU/cell with the plaque-purified recombinant virus (from Subheading 3.3, **step 9**) in 7 mL of RPMI-GlutaMAX/2.5% FBSΔ per flask. Incubate on a rocking platform for adsorption and entry at 37 °C for 1.5 h.
4. Remove the viral inoculum and add 20–25 mL of RPMI-GlutaMAX/2.5% FBSΔ per flask. Incubate at 37 °C.
5. Observe the flasks daily for the presence of cytopathic effect and the expression of a fluorescent reporter, if applicable (e.g., EGFP as in the case of R-115).
6. Two days after infection, check for full cytopathic effect (rounded up or detached cells, *see* **Note 7**). Collect infected cell medium and detached cells with a cell scraper. Distribute the suspension in 50 mL conical tubes.
7. Spin down cells and debris by low-speed centrifugation, at 2000 × *g* for 15–20 min at 4 °C.

8. To pellet the virions, spin supernatant at 23,000 × *g* for 1 h 10 min (*see* **Note 8**).
9. Remove carefully the supernatant, paying attention not to touch or displace the virion pellet. Leave about 50 μL of medium exactly on the virion pellet and let the tubes stand on ice for about 30 min (*see* **Note 9**).
10. Resuspend the virion pellet by gentle pipetting (avoid vortexing). Make small volume aliquots (50–100 μL) and store at −80 °C. Thaw one aliquot and titer (*see* Subheading 3.5).

3.4 Purification of Virions by Iodixanol Gradient

This purification protocol includes an additional filtration step in order to separate the recombinant virions from cellular debris. The ultracentrifugation is followed by an iodixanol gradient, where the purified virions form a band which can be rescued. Purity is improved at the expense of yield: the recovery of virions ranges from 40% to 60% (*see* **Note 5**).

1. Seed 11 T175 with 1.8×10^7 SK-OV-3 cells in 25 mL RPMI-GlutaMAX/10% FBSΔ per flask and incubate overnight at 37 °C in a CO_2 incubator (*see* **Note 6**). Allow cells to become 80–100% confluent (it is not recommended to let cells become over confluent).
2. Trypsinize one T175 flask and determine the cell number. Proceed if it is in the range of 1.8–2 × 10^7 cells/T175.
3. Infect the 10 T175 at MOI 0.1 PFU/cell with plaque-purified recombinant virus (from Subheading 3.3, **step 9**) in 7 mL RPMI-GlutaMAX/2.5% FBSΔ per flask. Incubate on a rocking platform for adsorption and entry at 37 °C for 1.5 h.
4. Remove the viral inoculum and add 25 mL of RPMI-GlutaMAX/2.5% FBSΔ per flask. Incubate overnight at 37 °C.
5. The next day, move the flasks at 33 °C and incubate for 4 additional days (total time of infection: 5 days) (*see* **Note 10**).
6. Observe the flasks daily for the presence of cytopathic effect and the expression of a fluorescent reporter, if applicable.
7. Five days postinfection, check for full cytopathic effect (rounded up or detached cells, *see* **Note 7**). Detach cells with a scraper. Harvest infected cell medium and cells. Distribute the suspension in 50-mL conical tubes.
8. Spin down cells and debris by low-speed centrifugation, at 2000 × *g* for 15–20 min at 4 °C.
9. Filter the supernatant through a sterile filter unit with cellulose nitrate membrane, 0.8 μm pore size.
10. Meanwhile sterilize six 38.5-mL ultracentrifugation tubes: fill with 70% ethanol, let stand for 15 min, wash five times with sterile ddH_2O and dry under laminar flow hood.

11. Transfer 1.5 mL of 50% iodixanol WS (solution C) into the tubes (iodixanol cushion).
12. Carefully and very slowly, paying attention not to perturb the iodixanol cushion, fill completely each tube with the filtered supernatant from **step 9**, usually 37 mL (*see* **Note 11**).
13. Centrifuge at 121,300 × *g* (max RCF) for 2 h to concentrate the virus at the cushion–medium interface. Let the rotor stop without brake.
14. Without disturbing the iodixanol cushion and the virus at the interface, remove the upper layer (usually 35–36 mL), that is, leave in the tube a volume equal to the volume of the cushion (1.5 mL).
15. Mix the residual content of the tube. This will result in a concentrated virus suspension in about 25% (w/v) of iodixanol (solution E).
16. Distribute the suspension (usually about 18 mL) into smaller 5.1-mL ultracentrifuge quick seal tubes and fill, if necessary, with 25% iodixanol (solution E).
17. Centrifuge at 199′000 × *g* (max RCF) overnight using a vertical rotor, without brake for deceleration.
18. Secure the tubes on a metal stand (cannula) and harvest the recombinant virus band with a syringe. Make small aliquots (50–100 μL), store at −80 °C. Thaw one aliquot and determine the recombinant virus titer (*see* Subheading 3.5).

3.5 Titration by Plaque Assay

Titration of the recombinant virus preparations is carried out in appropriate cells to determine the concentration of infectious viral particles (as plaque-forming units [PFU]/mL). The protocol below refers to a titration of R-115 in SK-OV-3 cell line.

1. Seed 5×10^5 cells in 1 mL RPMI-GlutaMAX/10% FBSΔ per well of a 12-well cell culture plate (*see* **Note 12**). Incubate overnight at 37 °C in a CO_2 incubator.
2. Prepare tenfold serial dilutions of recombinant virus in low serum medium (RPMI-GlutaMAX/2.5% FBSΔ), in the 10^{-2}–10^{-8} range (*see* **Note 13**).
3. Remove the medium from the wells of the 12-well plate and infect the cell monolayers with 350 μL of the virus dilutions. Incubate at 37 °C for 1.5 h on a rocking platform for adsorption and infection.
4. Prepare the agarose overlay medium a few minutes before the end of the virus adsorption period and keep it at 45 °C to prevent solidification (*see* **Note 3**). Replace the virus inoculum with 1 mL/well of the agarose overlay. Keep the plates at RT

for 20 min to allow the agarose solidify. Incubate at 37 °C for 4–5 days in a CO_2 incubator.

5. Score the number of plaques using a microscope or stereomicroscope. If the virus expresses a fluorescent marker, use a fluorescence microscope with the appropriate filters to score the number of plaques. Only wells containing 10–100 plaques are counted. Virus titer is expressed as plaque-forming units (PFU) per mL (*see* **Note 14**).

   If the plaques are not easily detectable (e.g., they are too small), it is possible to perform an immunostaining of the infected cell monolayers. In this case the agarose overlay must be avoided, and replaced with other overlays suitable for plaques formation. After **steps 1–3**, proceed with **step 6**.

6. Add the appropriate amount of neutralizing antibody to low serum medium (e.g., RPMI-GlutaMAX/2.5% FBSΔ). For R-115, 52S ascites (anti gH) is used at 1:10,000 dilution. Alternatively, medium supplemented with 0.67% methyl cellulose can be used. Replace the virus inoculum with 1 mL/well of medium with antibody, and incubate at 37 °C for 4–5 days in a CO_2 incubator (*see* **Note 3**).

7. Remove the medium and fix the cell monolayers with 500 µL of a fixing reagent (e.g., methanol at −20 °C for 10 min, 4% paraformaldehyde in PBS at RT, or other reagents) (*see* **Note 15**). Wash the cell monolayers twice with 1 mL PBS.

8. To carry out the staining of an intracellular antigen, after fixing with crosslinking reagents (e.g., paraformaldehyde) an extra step is added to permeabilize the cells: incubate with PBS + 0.1–1% Triton X-100 for 20 min, then wash twice with 1 mL PBS. This step can be avoided following fixing with alcohols, that simultaneously fix and permeabilize cells, or if the target antigen is displayed on the cell surface.

9. Primary antibody: incubate the cell monolayers with 350 µL PBS + 20% FBS + primary antibody. For R-115 recombinant virus, a 1:500 dilution of 52S antibody is used (*see* **Note 15**). Incubate for 60 min at RT. Wash the cell monolayers twice with 1 mL PBS.

10. Secondary antibody: incubate the cell monolayers with 350 µL PBS + 20% FBS + diluted secondary antibody (e.g., anti-mouse FITC) for 60 min. Wash the cell monolayers twice with 1 mL PBS.

11. Following fluorochrome-conjugated secondary antibody staining, use a fluorescence microscope with the appropriate filters to score the number of plaques (as above, count wells containing 10–100 plaques). Following an incubation with an alkaline phosphatase-conjugated secondary antibody, wash cell monolayers with 1 mL of AP buffer, then add 350 µL of AP

substrate. Incubate at 37 °C for 30 min for a violet/gray stain to develop, stop by washing with PBS and score the number of plaques and calculate infectious recombinant virus titer (*see* **Note 14**).

3.6 Titration by qPCR: Determination of Viral Genome Copies

Viral particles can be titrated also by determination of the genome copies (gc). From this value it is possible to calculate the gc/PFU ratio. This parameter provides an estimate of the infectious to encapsidated/enveloped noninfectious viral particles present in the recombinant virus preparation. The ratio obtained for a certain recombinant virus relative to that of the wild type virus is an indirect indication of the amount of defective viral particles. Clearly, the procedure illustrated below can be modified relative to the DNaseI treatment and/or the employment of detergent in the resuspension buffer. For example, by omitting the DNaseI treatment, one can obtain a measure of the amount of unencapsidated/uneveloped viral DNA. The protocol below refers to a titration of R-115.

1. Dilute virions 1:100 in resuspension buffer and add 50 U of DNaseI to 100 μL of the dilution. Incubate 30 min at 37 °C. This step digests the nonencapsidated recombinant virus genomes, and enables the gc quantification for encapsidated virions only.
2. Stop the DNaseI digestion by adding 5 μL of 0.5 M EDTA and incubating at 80 °C for 20 min.
3. Add 45 μL of 0.2% SDS and 5 μL of 20 μg/μL Proteinase K to 50 μL of the previous solution. Vortex and incubate for 1 h at 56 °C, then for 15 min at 95 °C. Viral DNA is released in solution.
4. Prepare tenfold serial dilutions of viral DNA in ddH_2O, in the 10^{-2}–10^{-4} range.
5. To make a standard curve, use ddH_2O to dilute spectrophotometrically quantified DNA of HSV-BAC 115 to 10^8 genomes/μL. Prepare tenfold serial dilutions in ddH_2O, to obtain 10^7–10^1 genomes/μL.
6. Use viral and HSV-BAC DNA dilutions in a qPCR reaction. Five μL of each dilution are used as template for reactions run in triplicate. For example, for R-115, a Taqman qPCR assay is performed, using the primers DnapolFw (CATCACCGACCCGGAGAGGGAC) (forward), DnapolRev (GGGCCAGGCGCTTGTTGGTGTA) (reverse), and DNA_Pol_PROBE (5′ FAM–3′ Tamra CCGCCGAACTGAGCAGACACCCGCGC), annealing to HSV UL30 ORF (DNA polymerase) [3].
7. Use the standard curve obtained with HSV-BAC DNA (ct *vs* genome copies) to interpolate the values obtained for the serial

dilutions of virions. Calculate the average of values obtained from the 10^{-2}–10^{-4} dilutions. Express values as gc/mL, and divide by the titer expressed as PFU/mL. Calculate the gc/PFU ratio (*see* **Note 16**).

3.7 Detection of Transgene Expression

This assay allows the detection of the transgenic protein encoded by the recombinant virus. The following protocol refers to an assay in SK-OV-3 cell line.

1. Seed a 12-well cell culture plate with 5×10^5 cells in 1 mL RPMI-GlutaMAX/10% FBSΔ (*see* **Note 12**). Incubate o/n at 37 °C in a CO_2 incubator.
2. Infect the cell monolayers with the recombinant virus expressing the transgene (e.g., R-115 engineered to encode mIL12) or with the control recombinant virus (e.g., R-LM113, same backbone, but no transgene) at 0.1–1 PFU/cell in 350 μL of low serum medium. Incubate at 37 °C for 90 min on a rocking shaker.
3. Replace the virus inoculum with 1.5 mL of low serum medium. Incubate plates at 37 °C for 3 days in a CO_2 incubator.

Follow **steps 4** and **5** for detection of transgene expression by ELISA.

4. At 24, 48, and 72 h postinfection, withdraw an appropriate volume (150–300 μL) of culture medium from each well, pellet and discard any cell, recover the supernatant and snap freeze in liquid nitrogen to avoid protein degradation.
5. Proceed with transgenic protein quantification, using a commercial or in house ELISA kit. Express the concentration of secreted protein as pg/mL. For example, to quantify mIL12 secreted by cells infected with R-115 recombinant virus, 150 μL of medium are taken from wells infected with R-115 or R-LM113 (control), at 24, 48, and 72 h after infection. 50 μL of each sample is used in ELISA, in duplicate, following the manufacturer's instructions. To eliminate matrix effect on the values, averages of the replicates of the mIL12-positive recombinant virus R-115 are subtracted of mIL12 background values detected in the medium of the control mIL12-negative recombinant virus (e.g., R-LM113 [4]).

Follow **steps 6–10** for detection of transgene expression by flow cytometry.

6. At 24, 48, and 72 h postinfection, remove the medium and detach cells using a scraper, or by trypsinization.
7. Pellet cells at 400 × *g* for 7 min, then resuspend the pellet in 50 μL of ice-cold FACS buffer to dissociate any clump. Keep cells on ice for the rest of the experiment.

8. React cells with the appropriate dilution of fluorochrome-conjugated antibody directed against the transgenic product. Keep a sample unstained as negative control. Incubate on ice for 30 min (*see* **Note 17**).
9. Wash cells twice with 1 mL of FACS buffer, pelleting at 400 × *g* for 7 min. Resuspend pellets in 300 μL of FACS buffer.
10. Acquire the sample data by flow cytometry ($1–5 \times 10^4$ events in the gate, per sample) with the appropriate filters. Express the data as the mean intensity of the fluorescence signal of the stained cells, after subtraction of the mean fluorescence intensity of the cells reacted with the secondary antibody only.

Follow **steps 11–13** for detection of transgene expression by Western blot.

11. For a secreted transgene product, at 24, 48, and 72 h postinfection, take an aliquot of the medium and proceed to **step 13**. For an intracellular or cell-associated transgene product, remove the medium and lyse cells with 200 μL lysis buffer. Incubate on ice for 15 min. Pellet cell debris at 11,000 × *g* for 10 min and discard the pellets.
12. Measure protein concentration in the media or supernatants by either direct fluorescence determination, Bradford or BCA, using a standard curve with known concentrations of bovine serum albumin.
13. Use the same amount of proteins (in the range of 10–250 μg) or the same volume of medium (for secreted proteins) for SDS polyacrylamide gel electrophoresis (SDS PAGE). Transfer the proteins to a nitrocellulose or PVDF membrane and detect transgenic product and control proteins (e.g., tubulin or β-actin) with appropriate antibodies. Develop WB with ECL reagents, detect and quantify signals as appropriate. For a qualitative assay, compare cell lysates infected by transgene-expressing or -non-transgene-expressing viruses. For a semiquantitative analysis, use known amounts of the purified transgenic product to create a standard curve in the blot. Use the curve to calculate the amount of transgenic product expressed by the infected cells.

3.8 Detection of Transgene mRNA Expression by qRT-PCR

1. At 24, 48, and 72 h postinfection, remove the medium and extract total RNA with a commercially available kit, according to the manufacturer's instructions. Determine RNA concentration with an UV spectrophotometer.
2. Use 2 μg of RNA for cDNA synthesis, with a retrotranscription kit, according to the manufacturer's instructions.
3. Dilute the cDNAs in ddH_2O (1:5) and use 2 μL in a qRT-PCR reaction. For the quantification of transgenic mIL12 expressed

from R-115-infected cells, a qRT-PCR assay is performed, using the probes for mIL12 (Mm00434169_m1) and for a housekeeping gene of SK-OV-3 cells (human gapdh, Taqman assay Hs99999905_m1). Calculate results by means of Δct method, comparing the expression of mIL12 in cells infected with R-115- or the control mIL12-negative R-LM113 recombinant virus.

3.9 Extent of Infection

1. Seed a 24-well cell culture plate with the cell lines of choice (*see* **Note 12**). Incubate at 37 °C in a CO_2 incubator.
2. Infect cells at 2–10 PFU/cell with the recombinant and the wt control virus, or mock-infect. Infections are carried out in 200 μL of low serum medium. Incubate at 37 °C for 90 min on a rocking platform.
3. Replace the viral inoculum with 500 μL of fresh low serum medium. Incubate for 24–48 h at 37 °C in a CO_2 incubator.
4. If the recombinant virus expresses a fluorescent marker, monitor infection by fluorescence microscopy with the appropriate filters. Otherwise, an immunostaining can be performed (*see* Subheading 3.5, **step 5**).
5. To quantitatively measure the infected cells, analyze samples by flow cytometry. After **steps 1–3**, remove the medium and detach cells using a scraper, or trypsin–EDTA.
6. Pellet cells at 400 × *g* for 7 min, then resuspend the pellet in 50 μL of ice-cold FACS buffer to dissociate any clumps. Keep cells on ice for the rest of the experiment.
7. If recombinant virus expresses a fluorescent marker, go to **step 9**. Otherwise, select a virus-expressed protein, which is localized on the surface of the cell, and use the appropriate amount of fluorochrome-conjugated antibody directed against this protein (*see* **Note 17**). Incubate on ice for 30 min.
8. Wash cells two times with 1 mL of FACS buffer, pelleting at 400 × *g* for 7 min. Resuspend in 300 μL of FACS buffer.
9. Acquire the sample with a flow cytometer with appropriate filters for the fluorochrome (1–5 × 10^4 events in the gate, per sample). Use the signal of the mock-infected cells to set the "zero" of the fluorescence, and express the infection as the percentage of infected cells.

3.10 Extent of Recombinant Virus Replication

The protocol below refers to an assay carried out in SK-OV-3 cells to measure the kinetic of recombinant virus production in infected cells.

1. Seed 12-well cell culture plates with 5 × 10^5 cells in 1 mL RPMI-GlutaMAX/10% FBSΔ (*see* **Note 12**). Incubate at 37 °C in a CO_2 incubator overnight. The number of plates

corresponds to the time points to be analyzed (usually at least two, for 24 and 48 h).

2. Infect cells at 0.1–1 PFU/cell. Infections are carried out in 350 μL of low serum medium (RPMI-GlutaMAX/2.5% FBSΔ). Incubate for adsorption and entry at 37 °C for 90 min on a rocking shaker.
3. To inactivate unpenetrated recombinant virus, wash once with PBS, then perform an acidic wash (pH 3 wash) for 1 min. Wash twice with 1 mL of PBS. Then, add 1 mL/well of low serum medium. Incubate at 37 °C in a CO_2 incubator.
4. Block the infections at the chosen time points (24 and 48 h) by freezing the plate at −80 °C.
5. Seed 12-well plates for titration (*see* Subheading 3.5).
6. Thaw the frozen plates on ice, scrape the bottom of each well and collect the medium with the cell lysate in 2 mL Eppendorf tubes.
7. Sonicate the content of the tubes to release the viral particles from the cells.
8. Perform titration with serial dilutions as described (*see* Subheading 3.5). Express results as PFU/mL or PFU/cell at 24 and 48 h.

4 Notes

1. When handling HSV-BAC DNA always use wide-orifice tips to prevent DNA fragmentation.
2. For transfection of SK-OV-3 cells, which grow in medium supplemented with 10% FBSΔ, serum is reduced to 2.5%. For other cell lines, that normally require media containing 5% FBS, serum may be reduced to 1% FBS.
3. The agarose layer blocks the diffusion of progeny virus in the medium and allows viral spread only to adjacent cells. At suitable dilutions, every plaque derives from a single virion present in the initial inoculum. Methyl cellulose has the same mechanical effect on virus progeny diffusion. An equivalent result can be obtained with neutralizing antibodies, which block progeny virus released in the medium.
4. Infected cells display rounding (cytopathic effect, c.p.e.). At late stages of infection they lyse. This phenomenon causes the light passing through infected cells to refract differently than the surrounding uninfected cells, and the plaque can be visualized under the stereomicroscope as a darker zone, with possibly a small hole at the center. If you do not feel confident, and if the recombinant virus expresses a suitable reporter (e.g., EGFP

as in R-115), before picking up the plaques you can check under the fluorescence microscope.

5. Take aliquots during the purification process for analysis and titration to monitor recovery at every step.
6. This procedure is devised for the preparation of virions from 10 T175 flasks of SK-OV-3 cells. The protocol can be scaled up or down depending on specific needs.
7. SK-OV-3 cells never detach completely from the flask, but all cells should be rounded up.
8. Higher *g*-force can make virion resuspension difficult.
9. This will allow the virion pellet to resuspend more easily. It is pivotal to avoid drying of the pellet during the incubation on ice.
10. For the production of recombinant virions, which may replicate more slowly than wt virus, lowering the temperature to 33 °C slows down cell growth allowing more time to recombinant virus replication and avoiding cells outgrowing the virus. After 5 days, cells will look strongly altered, but will nevertheless give a good virus yield. For every combination of recombinant virus and host cell line, it is worth comparing the recombinant virus growth in standard conditions (2–3 days at 37 °C) with the low temperature conditions (1 day at 37 °C followed by 4 days at 33 °C).
11. To speed up the process, you can also first fill the tube with supernatant and afterward add quickly 1.5 mL of 50% iodixanol WS going at the bottom of the tube.
12. Seed the wells with a number of cells suitable to achieve 100% confluency after an overnight incubation. Do not exceed with the number of cells: infection and titration assays performed in highly dense monolayers can lead to underestimation of the actual recombinant virus titer.
13. According to the expected titer and the quantity of recombinant virus available, serial dilutions may start from 10^{-1}, made by adding 50 μL of recombinant virus to 450 μL of low serum medium. For small amounts of concentrated recombinant virus (usually virions) the first dilution is 10^{-2}, made by adding 5 μL of recombinant virus to 495 μL of low serum medium. All the subsequent tenfold dilutions are prepared by adding 50 μL of the previous dilution to 450 μL of low serum medium.
14. Calculation of the titer: number of plaques (PFU) $\times$ $10^{(-\text{dilution})}/0.35$ mL. For example, 23 plaques in dilution -8 correspond to $23\ \text{PFU} \times 10^8/0.35\ \text{mL} = 6.6 \times 10^9$ PFU/mL. For accuracy and statistical significance, the titrations should be carried out in duplicate or triplicate.

15. The choice of fixing solution depends on the primary antibody to be used for the immunostaining. For different antigens, the optimal combination of fixing conditions and working antibody concentration must be determined by the operator.
16. Recombinant virion preparations are typically in the range of 200–300 gc/PFU in SK-OV-3, which means one infectious virion every 200–300 virions. This value indicates a great prevalence of nonencapsidated genomes (>99.5%) over the infectious virions.
17. As an alternative to a fluorochrome-conjugated antibody directed against the transgene product, it is possible to use a primary antibody directed to the antigen of interest, followed by a fluorochrome-conjugated secondary antibody. The optimal working antibody concentrations must be determined by the operator.

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