

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Andrea Vannini, B.P. (2020). Rescue, Purification, and Characterization of a Recombinant HSV Expressing a Transgenic Protein. New York, NY : Humana Press [10.1007/978-1-4939-9814-2_8].

Availability:

This version is available at: <https://hdl.handle.net/11585/708240> since: 2019-12-13

Published:

DOI: http://doi.org/10.1007/978-1-4939-9814-2_8

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Vannini A, Petrovic B, Gatta V, Leoni V, Pepe S, Menotti L, et al. Rescue, Purification, and Characterization of a Recombinant HSV Expressing a Transgenic Protein.

In: Diefenbach RJ, Fraefel C, ed. Herpes Simplex Virus New York, NY: Springer New York; 2020 pag. 153–68. (Methods in Molecular Biology; vol. 2060).

The final published version is available online at:

https://doi.org/10.1007/978-1-4939-9814-2_8

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

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

Andrea Vannini, Biljana Petrovic, Valentina Gatta, Valerio Leoni,
Simona Pepe, Laura Menotti, Gabriella Campadelli-Fiume, and
Tatiana Gianni

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”).	29
	30
2. HSV-1 BAC DNA (e.g., R-115; [1]).	31
3. Phosphate buffered saline (PBS): 9.0 g NaCl, 795 mg Na ₂ HPO ₄ ·H ₂ O, 144 mg KH ₂ PO ₄ in 800 mL ddH ₂ O, adjust pH to 7.4, make up to 1 L with ddH ₂ O, sterilize, store at 4 °C.	32
	33
	34
4. Trypsin–EDTA (0.05%) in PBS without calcium and magnesium.	35
	36
5. 2× DMEM.	37
6. 5% agarose stock solution: dissolve 5 g of cell culture grade Sea Plaque Agarose (Lonza) in 100 mL ddH ₂ O, autoclave for 20 min at 121 °C. While still hot, mix well. Store at room temperature.	38
	39
	40
	41
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.	42
	43
	44
	45
	46
	47
	48
8. Lipofectamine 2000 (Life Technologies).	49
9. Acidic wash solution: 40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3; autoclave.	50
	51
10. 70% ethanol.	52
11. 0.67% methyl cellulose (4000 centipoise) in RPMI-GlutaMAX/6% FBSΔ.	53
	54
	55
2.2 Iodixanol solutions	
1. Solution A. OptiPrep Sigma [60% (w/v) solution of iodixanol in water, sterile].	56
	57
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 ₂ O. Sterilize through a 0.2 μm filter.	58
	59
	60
	61
3. Solution C: 50% iodixanol WS (working solution, for cushion): mix 5 volumes of OptiPrep (Solution A) with 1 volume of Diluent (Solution B).	62
	63
	64
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 ₂ O. Sterilize through a 0.2 μm filter.	65
	66
	67
	68
5. Solution E: 25% iodixanol: mix equal volumes of 50% iodixanol (Solution C) and HEPES buffered saline (Solution D).	69
	70
	71

2.3 Determination of Viral Genome Copies by qPCR

1. DNaseI. 72
2. Resuspension buffer: 10 mM Tris-HCl, 75 mM NaCl, 1 mM MgCl₂, 0.02% PS-80, 5% sucrose, 0.1 mM EDTA, 10 mM L-histidine, 0.5% ethanol, pH 7.4. 73
74
75
3. 0.5 M EDTA (pH 8.0): 186.1 g Na₂EDTA·2H₂O in 800 mL ddH₂O, adjust pH to 8.0 with NaOH, make up to 1 L with ddH₂O, aliquot and sterilize by autoclaving. 76
77
78
4. 0.2% SDS in ddH₂O. 79
5. Proteinase K, 20 µg/mL in ddH₂O. 80
81

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

1. Liquid nitrogen. 82
2. Fixing solution (e.g., methanol, 4% paraformaldehyde, ethanol). 83
84
3. 0.1%/1% Triton X-100 in ddH₂O. 85
4. Diluent for antibodies (immunostaining): 20% FBS in PBS. 86
5. AP buffer: 100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 9.6. 87
88
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. 89
90
91
7. FACS buffer: 2% FBS in PBS. 92
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. 93
94
95
96
97

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]. 98
99
100
101
102
2. Secondary antibodies: anti-mouse and anti-rabbit Alexa Flour 488-conjugated antibodies. 103
104
105

2.6 Kits

1. Genomic DNA extraction kit. 106
2. Mouse IL-12 p70 ELISA kit (Thermo Scientific or R&D). 107
3. ECL Western blotting detection kit. 108
4. Reverse transcription kit. 109
110

2.7 Equipment

1. Bench instrumentation: benchtop centrifuges, microplate reader, Western Blot apparatus and immunodetection system, ultrasound sonicator, spectrophotometer, water bath, rocking platform. 111
112
113
114

2. Microscopes: fluorescence microscope, inverted microscope, stereomicroscope.	115 116
3. Molecular biology: PCR and RealTime-PCR thermal cyclers, gel electrophoresis system, gel imaging system.	117 118
4. CO ₂ incubator for mammalian cell cultures.	119
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.	120 121 122
6. T25 and T175 tissue culture flasks.	123
7. 6-, 12-, and 24-well tissue culture plates.	124
8. 1.5 and 2 mL Eppendorf tubes.	125
9. 50 mL conical tubes.	126
10. Pasteur pipettes.	127
11. Cell scrapers.	128
12. 0.8 µm cellulose nitrate membrane filters.	129
13. Nitrocellulose or PVDF membranes.	130 131

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.	133
Transfect at least four recombinant clones, in duplicate.	134
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.	135 136 137 138
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.	139 140 141
3. Meanwhile, dilute 0.5–1 µg of quantified HSV-BAC DNA in 100 µL of medium without antibiotics and serum (<i>see Note 1</i>).	142 143
4. Mix gently together the diluted DNA and Lipofectamine 2000, and incubate at room temperature for 20 min.	144 145
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 (<i>see Note 2</i>).	146 147 148 149
6. After the incubation time, gently transfer drop-by-drop the DNA–Lipofectamine mix on cells using a pipette with wide-orifice tip.	150 151 152
7. Incubate cells for 2–3 days at 37 °C in a CO ₂ incubator. No medium replacement is needed following transfection.	153 154

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 $-80\text{ }^{\circ}\text{C}$ 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 $12\text{ }\mu\text{m}$ peak to peak. Keep frozen at $-80\text{ }^{\circ}\text{C}$ 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% FBS Δ . Incubate overnight at $37\text{ }^{\circ}\text{C}$ in a CO_2 incubator.
10. Thaw the Seed lysate at $37\text{ }^{\circ}\text{C}$ 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 $37\text{ }^{\circ}\text{C}$ for 1.5 h.
11. Remove inoculum, add RPMI-GlutaMAX/2.5% FBS Δ 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% FBS Δ . Incubate overnight at $37\text{ }^{\circ}\text{C}$ in a CO_2 incubator and allow cells to become confluent.
2. Infect monolayers with $350\text{ }\mu\text{L}$ of tenfold dilutions of recombinant virus from passage 1 (p1). Place the plate on rocking platform at $37\text{ }^{\circ}\text{C}$ for 1.5 h. After virus adsorption, replace the viral inoculum with agarose overlay (*see* **Note 3**). Incubate at $37\text{ }^{\circ}\text{C}$ 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 $500\text{ }\mu\text{L}$ 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 $350\text{ }\mu\text{L}$ of the undiluted plaque medium and $350\text{ }\mu\text{L}$ of three tenfold dilutions, from 10^{-1} to 10^{-3} . Place the plate on rocking platform at $37\text{ }^{\circ}\text{C}$ for 1.5 h. Store the rest of the undiluted plaque medium at $-80\text{ }^{\circ}\text{C}$.
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 ($\sim 10^5$ cells/cm²) 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 μ L 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% FBS Δ per flask and incubate overnight at 37 °C in a CO₂ 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 $1.8\text{--}2 \times 10^7$ 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% FBS Δ 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% FBS Δ 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 $2000 \times g$ for 15–20 min at 4 °C.

8. To pellet the virions, spin supernatant at $23,000 \times g$ for 1 h 244
10 min (*see Note 8*). 245
9. Remove carefully the supernatant, paying attention not to 246
touch or displace the virion pellet. Leave about 50 μL of 247
medium exactly on the virion pellet and let the tubes stand on 248
ice for about 30 min (*see Note 9*). 249
10. Resuspend the virion pellet by gentle pipetting (avoid vortex- 250
ing). Make small volume aliquots (50–100 μL) and store at 251
 -80°C . Thaw one aliquot and titer (*see Subheading 3.5*). 252
253

3.4 Purification of Virions by Iodixanol Gradient

This purification protocol includes an additional filtration step in 254
order to separate the recombinant virions from cellular debris. The 255
ultracentrifugation is followed by an iodixanol gradient, where the 256
purified virions form a band which can be rescued. Purity is 257
improved at the expense of yield: the recovery of virions ranges 258
from 40% to 60% (*see Note 5*). 259

1. Seed 11 T175 with 1.8×10^7 SK-OV-3 cells in 25 mL RPMI- 260
GlutaMAX/10% FBS Δ per flask and incubate overnight at 261
 37°C in a CO_2 incubator (*see Note 6*). Allow cells to become 262
80–100% confluent (it is not recommended to let cells become 263
over confluent). 264
2. Trypsinize one T175 flask and determine the cell number. 265
Proceed if it is in the range of $1.8\text{--}2 \times 10^7$ cells/T175. 266
3. Infect the 10 T175 at MOI 0.1 PFU/cell with plaque-purified 267
recombinant virus (from Subheading 3.3, step 9) in 7 mL 268
RPMI-GlutaMAX/2.5% FBS Δ per flask. Incubate on a rocking 269
platform for adsorption and entry at 37°C for 1.5 h. 270
4. Remove the viral inoculum and add 25 mL of RPMI-Gluta- 271
MAX/2.5% FBS Δ per flask. Incubate overnight at 37°C . 272
5. The next day, move the flasks at 33°C and incubate for 4 addi- 273
tional days (total time of infection: 5 days) (*see Note 10*). 274
6. Observe the flasks daily for the presence of cytopathic effect 275
and the expression of a fluorescent reporter, if applicable. 276
7. Five days postinfection, check for full cytopathic effect 277
(rounded up or detached cells, *see Note 7*). Detach cells with 278
a scraper. Harvest infected cell medium and cells. Distribute the 279
suspension in 50-mL conical tubes. 280
8. Spin down cells and debris by low-speed centrifugation, at 281
 $2000 \times g$ for 15–20 min at 4°C . 282
9. Filter the supernatant through a sterile filter unit with cellulose 283
nitrate membrane, 0.8 μm pore size. 284
10. Meanwhile sterilize six 38.5-mL ultracentrifugation tubes: fill 285
with 70% ethanol, let stand for 15 min, wash five times with 286
sterile ddH₂O and dry under laminar flow hood. 287

11. Transfer 1.5 mL of 50% iodixanol WS (solution C) into the tubes (iodixanol cushion). 288
289
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*). 290
291
292
13. Centrifuge at $121,300 \times g$ (max RCF) for 2 h to concentrate the virus at the cushion–medium interface. Let the rotor stop without brake. 293
294
295
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). 296
297
298
299
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). 300
301
302
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). 303
304
305
17. Centrifuge at $199,000 \times g$ (max RCF) overnight using a vertical rotor, without brake for deceleration. 306
307
18. Secure the tubes on a metal stand (cannula) and harvest the recombinant virus band with a syringe. Make small aliquots (50–100 μ L), store at -80°C . Thaw one aliquot and determine the recombinant virus titer (*see Subheading 3.5*). 308
309
310
311

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. 313
314
315
316

1. Seed 5×10^5 cells in 1 mL RPMI-GlutaMAX/10% FBS Δ per well of a 12-well cell culture plate (*see Note 12*). Incubate overnight at 37°C in a CO_2 incubator. 317
318
319
2. Prepare tenfold serial dilutions of recombinant virus in low serum medium (RPMI-GlutaMAX/2.5% FBS Δ), in the 10^{-2} – 10^{-8} range (*see Note 13*). 320
321
322
3. Remove the medium from the wells of the 12-well plate and infect the cell monolayers with 350 μ L of the virus dilutions. Incubate at 37°C for 1.5 h on a rocking platform for adsorption and infection. 323
324
325
326
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 327
328
329
330

- for 20 min to allow the agarose solidify. Incubate at 37 °C for 331
4–5 days in a CO₂ incubator. 332
5. Score the number of plaques using a microscope or stereomi- 333
croscope. If the virus expresses a fluorescent marker, use a 334
fluorescence microscope with the appropriate filters to score 335
the number of plaques. Only wells containing 10–100 plaques 336
are counted. Virus titer is expressed as plaque-forming units 337
(PFU) per mL (*see Note 14*). 338
If the plaques are not easily detectable (e.g., they are too 339
small), it is possible to perform an immunostaining of the 340
infected cell monolayers. In this case the agarose overlay must 341
be avoided, and replaced with other overlays suitable for pla- 342
ques formation. After **steps 1–3**, proceed with **step 6**. 343
 6. Add the appropriate amount of neutralizing antibody to low 344
serum medium (e.g., RPMI-GlutaMAX/2.5% FBSΔ). For 345
R-115, 52S ascites (anti gH) is used at 1:10,000 dilution. 346
Alternatively, medium supplemented with 0.67% methyl cellu- 347
lose can be used. Replace the virus inoculum with 1 mL/well of 348
medium with antibody, and incubate at 37 °C for 4–5 days in a 349
CO₂ incubator (*see Note 3*). 350
 7. Remove the medium and fix the cell monolayers with 500 μL of 351
a fixing reagent (e.g., methanol at –20 °C for 10 min, 4% 352
paraformaldehyde in PBS at RT, or other reagents) (*see Note* 353
15). Wash the cell monolayers twice with 1 mL PBS. 354
 8. To carry out the staining of an intracellular antigen, after fixing 355
with crosslinking reagents (e.g., paraformaldehyde) an extra 356
step is added to permeabilize the cells: incubate with 357
PBS + 0.1–1% Triton X-100 for 20 min, then wash twice with 358
1 mL PBS. This step can be avoided following fixing with 359
alcohols, that simultaneously fix and permeabilize cells, or if 360
the target antigen is displayed on the cell surface. 361
 9. Primary antibody: incubate the cell monolayers with 350 μL 362
PBS + 20% FBS + primary antibody. For R-115 recombinant 363
virus, a 1:500 dilution of 52S antibody is used (*see Note 15*). 364
Incubate for 60 min at RT. Wash the cell monolayers twice with 365
1 mL PBS. 366
 10. Secondary antibody: incubate the cell monolayers with 350 μL 367
PBS + 20% FBS + diluted secondary antibody (e.g., anti-mouse 368
FITC) for 60 min. Wash the cell monolayers twice with 369
1 mL PBS. 370
 11. Following fluorochrome-conjugated secondary antibody stain- 371
ing, use a fluorescence microscope with the appropriate filters 372
to score the number of plaques (as above, count wells contain- 373
ing 10–100 plaques). Following an incubation with an alkaline 374
phosphatase-conjugated secondary antibody, wash cell mono- 375
layers with 1 mL of AP buffer, then add 350 μL of AP 376

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/unenveloped 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 ddH₂O, in the 10⁻²-10⁻⁴ range.
5. To make a standard curve, use ddH₂O to dilute spectrophotometrically quantified DNA of HSV-BAC 115 to 10⁸ genomes/µL. Prepare tenfold serial dilutions in ddH₂O, to obtain 10⁷-10¹ 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 (CATCACC-GACCCGGAGAGGGAC) (forward), DnapolRev (GGGCCAGGCGCTTGTGGTGTA) (reverse), and DNA_Pol_PROBE (5' FAM-3' Tamra CCGCCGAAGTGGAGAGCAGACACCCGCGC), 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% FBS Δ (*see Note 12*). Incubate o/n at 37 °C in a CO₂ 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 μ L 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 CO₂ 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 μ L) 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 μ L of medium are taken from wells infected with R-115 or R-LM113 (control), at 24, 48, and 72 h after infection. 50 μ L 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 $400 \times g$ for 7 min, then resuspend the pellet in 50 μ L 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 $400 \times g$ for 7 min. Resuspend pellets in 300 μ L 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 μ L lysis buffer. Incubate on ice for 15 min. Pellet cell debris at $11,000 \times g$ 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 μ g) 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 μ g of RNA for cDNA synthesis, with a retrotranscription kit, according to the manufacturer’s instructions.
3. Dilute the cDNAs in ddH₂O (1:5) and use 2 μ L in a qRT-PCR reaction. For the quantification of transgenic mL12 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 CO₂ 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 CO₂ 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 $400 \times g$ 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 $400 \times g$ for 7 min. Resuspend in 300 μL of FACS buffer.
9. Acquire the sample with a flow cytometer with appropriate filters for the fluorochrome ($1\text{--}5 \times 10^4$ 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% FBS Δ (*see Note 12*). Incubate at 37 °C in a CO₂ 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 μ L of low serum medium (RPMI-GlutaMAX/2.5% FBS Δ). 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 CO₂ 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% FBS Δ , 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 μ L of recombinant virus to 450 μ L of low serum medium. For small amounts of concentrated recombinant virus (usually virions) the first dilution is 10^{-2} , made by adding 5 μ L of recombinant virus to 495 μ L of low serum medium. All the subsequent tenfold dilutions are prepared by adding 50 μ L of the previous dilution to 450 μ L 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.

Acknowledgments

This work was supported by European Research Council (ERC) Advanced Grant number 340060, VII framework program to G. C.-F., by RFO (University of Bologna) to L.M. and T.G., and by Fondi Pallotti to T.G.

Competing interests: G.C.-F. owns shares in Nouscom Srl. B.P. is currently an employee of Nouscom Srl. G.C.-F. and L.M. receive equity payments from Amgen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Menotti L, Avitabile E, Gatta V, Malatesta P, Petrovic B, Campadelli-Fiume G (2018) HSV as A platform for the generation of retargeted, armed, and reporter-expressing oncolytic viruses. *Viruses* 10:pii:E352
2. Peng T, Ponce de Leon M, Novotny MJ, Jiang H, Lambris JD, Dubin G, Spear PG, Cohen GH, Eisenberg RJ (1998) Structural and antigenic analysis of a truncated form of the herpes simplex virus glycoprotein gH-gL complex. *J Virol* 72:6092–6103
3. Leoni V, Vannini A, Gatta V, Rambaldi J, Sanapo M, Barboni C, Zaghini A, Nanni P, Lollini PL, Casiraghi C, Campadelli-Fiume G (2018) A fully-virulent retargeted oncolytic HSV armed with IL-12 elicits local immunity and vaccine therapy towards distant tumors. *PLoS Pathog* 14:e1007209
4. Menotti L, Cerretani A, Hengel H, Campadelli-Fiume G (2008) Construction of a fully retargeted herpes simplex virus 1 recombinant capable of entering cells solely via human epidermal growth factor receptor 2. *J Virol* 20:10153–10161