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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 8 we describe the procedures to reconstitute, or regenerate, the replicating recombinant virus, and the 9 methods to purify it and characterize it for the correct expression of the transgene. We present the example 10 of R-115, a recombinant expressing murine interleukin 12 (mIL12) from the US1–US2 intergenic region. 11 A specific method for the production of highly purified virions by iodixanol gradient, suitable for in vivo 12 applications, is also detailed. 13

Key words HSV rescue, Plaque purification, Plaque assay, Virion purification, mIL12 transgene 14 expression 15

1 Introduction

In the previous chapter we describe the engineering by *galK* 17 recombineering in *E. coli* of an HSV-BAC carrying a transgenic 18 cassette, exemplified by the HSV-BAC 115 recombinant, engi-19 neered to encode murine IL12 (mIL12). Here we describe the 20 rescue of the recombinant virus R-115 [1] in susceptible and 21 permissive mammalian cells, the production of purified virions 22 suitable for animal experimentations, and the evaluation of the 23 transgenic mIL12 expression.

2 Materials

2.1 Cells, Cell Culture, and Transfection SK-OV-3 cells were purchased from ATCC and were grown in 26 RPMI Medium 1640-GlutaMAX-I with 100 units/mL peni- 27 cillin and 100 μg/mL streptomycin (hereafter "RPMI- 28

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	GlutaMAX"), supplemented with 10% heat-inactivated FBS (hereafter "FBS Δ ").	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 H-PO 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.	$2 \times \text{DMEM}.$	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 $2 \times DMEM$ 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 $2 \times DMEM$, 54 mL of RPMI-GlutaMAX, and 6 mL FBS Δ	42 43 44 45 46 47
	(6% final concentration). Keep at 45 °C until ready for use.	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-Gluta- MAX/6% FBSΔ.	53 54 55
2.2 Iodixanol 1. solutions	Solution A. OptiPrep Sigma [60% (w/v) solution of iodix anol 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_2O . 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 μ m 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 De	termination of	1. DNaseI.	72
Viral Ge by qPCR	Genome Copies PCR	 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 Na ₂ EDTA·2H ₂ O in 800 mL ddH ₂ O, adjust pH to 8.0 with NaOH, make up to 1 L with ddH ₂ O, aliquot and sterilize by autoclaving.	76 77 78
		4. 0.2% SDS in ddH ₂ O.	79
		5. Proteinase K, 20 μ g/mL in ddH ₂ O.	80 81
2.4 De	tection of	1. Liquid nitrogen.	82
Transgene Expression by ELISA, FACS, or	ne Expression A, FACS, or	2. Fixing solution (e.g., methanol, 4% paraformaldehyde, ethanol).	83 84
Western	Blot	3. 0.1%/1% Triton X-100 in ddH ₂ O.	85
		4. Diluent for antibodies (immunostaining): 20% FBS in PBS.	86
		5. AP buffer: 100 mM Tris, 100 mM NaCl, 5 mM MgCl ₂ , pH 9.6.	87 88
	6	6. Substrate for alkaline phosphatase: 330 μ g/mL NBT (nitroblue tetrazolium chloride) and 165 μ g/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
		 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 An	tibodies	 Primary antibodies: MAb R1.302 (gift from Dr. Marc Lopez, INSERM Marseille, or purchased from Santa Cruz Biotechnol- ogy) to nectin1; MAb 9G6 (Santa Cruz) to HER2; PAb R140 to HVEM (gift from Dr. Gary Cohen, University of Pennsyl- vania); MAb 52S to HSV gH [2]. 	98 99 100 101 102
		 Secondary antibodies: anti-mouse and anti-rabbit Alexa Flour 488-conjugated antibodies. 	103 104 105
2.6 Kit	s	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
2.7 Eq	uipment	1. Bench instrumentation: benchtop centrifuges, microplate reader, Western Blot apparatus and immunodetection system, ultrasound sonicator, spectrophotometer, water bath, rocking platform.	110 111 112 113 114

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
 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
	132

3 Methods

3.1 Rescue of Recombinant Virus (R-	The following protocol refers to transfection of SK-OV-3 cells. Transfect at least four recombinant clones, in duplicate.	133 134
115) from BAC-DNA by Transfection	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% FBS Δ . 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 μ L of Lipofectamine 2000 in 100 μ L of medium without antibiotics and serum, mix gently and incubate at room temperature for 5 min.	139 140 141
	 Meanwhile, dilute 0.5–1 μg of quantified HSV-BAC DNA in 100 μL of medium without antibiotics and serum (see Note 1). 	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</i> Note 2).	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 155 under the fluorescence microscope for the expression of EGFP 156 reporter. In case you find many plaques in a well, detach cells 157 with a scraper and transfer with the medium in a 2 mL Eppendorf tube. Sonicate or freeze at -80 °C to release intracellular 159 recombinant virus and make the Seed (the first virus culture to 160 be used to start amplified virus cultures). Sonication is set at 161 12 µm peak to peak. Keep frozen at -80 °C for long-term 162 storage. 163
- 9. Seed 1×10^6 SK-OV-3 cells per well of a 6-well tissue culture 164 plate in RPMI-GlutaMAX/10% FBS Δ . Incubate overnight at 165 37 °C in a CO₂ incubator. 166
- 10. Thaw the Seed lysate at 37 °C in a water bath and infect the 167 SK-OV-3 monolayer in the 6-well tissue culture plate from 168 step 9 with 1 mL of the Seed lysate per well. Place the plate 169 on a rocking platform at 37 °C for 1.5 h.
- 11. Remove inoculum, add RPMI-GlutaMAX/2.5% FBSΔ and 171 incubate for 2–3 days. This recombinant virus at passage 172 l (p1) will serve for further analysis (e.g., transgene sequenc-173 ing) and plaque purification (*see* Subheading 3.2).
- 1. Seed 5×10^5 SK-OV-3 cells per well of a 12-well tissue culture 176 plate in RPMI-GlutaMAX/10% FBS Δ . Incubate overnight at 177 37 °C in a CO₂ incubator and allow cells to become confluent. 178

175

- 2. Infect monolayers with 350 μ L of tenfold dilutions of recom- 179 binant virus from passage 1 (p1). Place the plate on rocking 180 platform at 37 °C for 1.5 h. After virus adsorption, replace the 181 viral inoculum with agarose overlay (*see* **Note 3**). Incubate at 182 37 °C in a CO₂ incubator for 3–5 days and monitor the 183 formation of plaques. 184
- The day before plaque picking, seed SK-OV-3 cells in 12-well 185 tissue culture plates as in step 1. In wells containing only a few 186 plaques, mark well-separated plaques under the stereomicro-187 scope or fluorescence microscope (*see* Note 4).
- 4. Pick at least four single plaques by pushing a sterile glass 189 Pasteur pipette through the agarose overlay. Transfer the aga- 190 rose plugs by pipetting up and down several times in 500 μ L 191 medium in a sterile 1.5 mL Eppendorf tube. Vortex and disrupt 192 the agarose plug by 15 s sonication. 193
- 5. Infect SK-OV-3 cells with 350 μ L of the undiluted plaque 194 medium and 350 μ L of three tenfold dilutions, from 10^{-1} to 195 10^{-3} . Place the plate on rocking platform at 37 °C for 1.5 h. 196 Store the rest of the undiluted plaque medium at -80 °C. 197
- 6. Carry out two additional rounds of plaque purification (steps 198 3–5). 199

3.2 Plaque Purification

- 7. After the third round of plaque purification, infect monolayers
of SK-OV-3 seeded in 6-well tissue culture plates or T25 flasks
 $(\sim 10^5 \text{ cells/cm}^2)$ to amplify the four plaque-purified recombi-
nant viruses. Incubate for 2 days.200
201
202
- 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 206 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). 210
- 10. Extract the DNA from 200 to 300 μL of infected cell lysate of 211 the selected plaque-purified recombinants by using a Genomic DNA Extraction kit. Confirm the presence of the transgene (e.g., mIL12) by molecular assays. 214

215

3.3 Concentration ofThe virion purification protocol entails a preliminary centrifugation216**Extracellular Virions**in order to concentrate extracellular virions from the infected cell217**by Ultracentrifugation**medium, with near 100% recovery (*see* Note 5). Additional down-218contaminating cellular DNA and proteins.220

- 1. Seed 11 T175 flasks with 1.8×10^7 SK-OV-3 cells in 25 mL221RPMI-GlutaMAX/10% FBS Δ per flask and incubate overnight222at 37 °C in a CO2 incubator (*see* Note 6). Allow cells to become22380–100% confluent (it is not recommended to let cells become224over confluent).225
- 2. Trypsinize one T175 flask and determine cell number. Proceed if it is in the range of $1.8-2 \times 10^7$ cells/T175. 227
- 3. Infect the 10 T175 flasks at MOI 0.5 PFU/cell with the plaque-purified recombinant virus (from Subheading 3.3, 229 step 9) in 7 mL of RPMI-GlutaMAX/2.5% FBSΔ per flask. 230 Incubate on a rocking platform for adsorption and entry at 37 °C for 1.5 h. 232
- 4. Remove the viral inoculum and add 20–25 mL of RPMI- 233 GlutaMAX/2.5% FBS Δ per flask. Incubate at 37 °C. 234
- 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 238 (rounded up or detached cells, *see* Note 7). Collect infected 239 cell medium and detached cells with a cell scraper. Distribute 240 the suspension in 50 mL conical tubes. 241
- 7. Spin down cells and debris by low-speed centrifugation, at 242 $2000 \times g$ for 15–20 min at 4 °C. 243

Characterization of Reconstituted HSV-BAC Recombinants

- 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 μ L 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 μ L) and store at 251 –80 °C. Thaw one aliquot and titer (*see* Subheading 3.5). 252

253

3.4 Purification of Virions by lodixanol 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 CO2 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 $1.8-2 \times 10^7$ cells/T175. ²⁶⁶
- 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% FBSΔ 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 additional 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
- 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	288
tubes (iodixanol cushion).	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).
 292
- 13. Centrifuge at $121,300 \times \mathcal{J}$ (max RCF) for 2 h to concentrate293the virus at the cushion-medium interface. Let the rotor stop294without brake.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).
 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).
- 16. Distribute the suspension (usually about 18 mL) into smaller 303
 5.1-mL ultracentrifuge quick seal tubes and fill, if necessary, 304 with 25% iodixanol (solution E). 305
- 17. Centrifuge at $199'^{000} \times g$ (max RCF) overnight using a vertical rotor, without brake for deceleration. 307
- 18. Secure the tubes on a metal stand (cannula) and harvest the 308 recombinant virus band with a syringe. Make small aliquots 309 $(50-100 \ \mu\text{L})$, store at $-80 \ ^{\circ}\text{C}$. Thaw one aliquot and determine the recombinant virus titer (*see* Subheading 3.5). 311

- **3.5 Titration by**Titration of the recombinant virus preparations is carried out in
appropriate cells to determine the concentration of infectious viral
appropriate cells to determine units [PFU]/mL). The protocol
below refers to a titration of R-115 in SK-OV-3 cell line.313
314
315
 - 1. Seed 5×10^5 cells in 1 mL RPMI-GlutaMAX/10% FBS Δ per317well of a 12-well cell culture plate (see Note 12). Incubate318overnight at 37 °C in a CO2 incubator.319
 - 2. Prepare tenfold serial dilutions of recombinant virus in low 320 serum medium (RPMI-GlutaMAX/2.5% FBS Δ), in the 321 10^{-2} - 10^{-8} range (*see* Note 13). 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. 326
 - 4. Prepare the agarose overlay medium a few minutes before the 327 end of the virus adsorption period and keep it at 45 °C to 328 prevent solidification (*see* Note 3). Replace the virus inoculum 329 with 1 mL/well of the agarose overlay. Keep the plates at RT 330

for 20 min to allow the agarose solidify. Incubate at 37 $^\circ$ 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).

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% FBS Δ). 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 μ L 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 μ L 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 μ L 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 staining, use a fluorescence microscope with the appropriate filters 372 to score the number of plaques (as above, count wells containing 10–100 plaques). Following an incubation with an alkaline 374 phosphatase-conjugated secondary antibody, wash cell monolayers 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 377 to develop, stop by washing with PBS and score the number of plaques and calculate infectious recombinant virus titer (*see* 379 Note 14). 380

380 381

Viral particles can be titrated also by determination of the genome 382 copies (gc). From this value it is possible to calculate the gc/PFU 383 ratio. This parameter provides an estimate of the infectious to 384 encapsidated/enveloped noninfectious viral particles present in 385 the recombinant virus preparation. The ratio obtained for a certain 386 recombinant virus relative to that of the wild type virus is an indirect 387 indication of the amount of defective viral particles. Clearly, the 388 procedure illustrated below can be modified relative to the DNaseI 389 treatment and/or the employment of detergent in the resuspension 390 buffer. For example, by omitting the DNaseI treatment, one can 391 obtain a measure of the amount of unencapsidated/uneveloped 392 viral DNA. The protocol below refers to a titration of R-115. 393

- 1. Dilute virions 1:100 in resuspension buffer and add 50 U of 394 DNaseI to 100 μ L of the dilution. Incubate 30 min at 37 °C. 395 This step digests the nonencapsidated recombinant virus gen- 396 omes, and enables the gc quantification for encapsidated 397 virions only. 398
- 2. Stop the DNaseI digestion by adding 5 μ L of 0.5 M EDTA and 399 incubating at 80 °C for 20 min. 400
- 3. Add 45 μ L of 0.2% SDS and 5 μ L of 20 μ g/ μ L Proteinase K to 401 50 μ L of the previous solution. Vortex and incubate for 1 h at 402 56 °C, then for 15 min at 95 °C. Viral DNA is released in 403 solution. 404
- 4. Prepare tenfold serial dilutions of viral DNA in ddH₂O, in the $405 10^{-2}$ - 10^{-4} range. 406
- 5. To make a standard curve, use ddH_2O to dilute spectrophoto- 407 metrically quantified DNA of HSV-BAC 115 to 10^8 genomes/ 408 µL. Prepare tenfold serial dilutions in ddH_2O , to obtain 409 10^7-10^1 genomes/µL. 410
- 6. Use viral and HSV-BAC DNA dilutions in a qPCR reaction. 411 Five μL of each dilution are used as template for reactions run 412 in triplicate. For example, for R-115, a Taqman qPCR assay is 413 performed, using the primers DnapolFw (CATCACC- 414 GACCCGGAGAGGGGAC) (forward), DnapolRev 415 (GGGCCAGGCGCTTGTTGGTGTA) (reverse), and DNA_- 416 Pol_PROBE (5' FAM-3' Tamra CCGCCGAACTGAGCAGA- 417 CACCCGCGC), annealing to HSV UL30 ORF (DNA 418 polymerase) [3]. 419
- 7. Use the standard curve obtained with HSV-BAC DNA (ct *vs* 420 genome copies) to interpolate the values obtained for the serial 421

3.6 Titration by qPCR: Determination of Viral Genome Copies

dilutions of virions. Calculate the average of values obtained 422 from the 10^{-2} – 10^{-4} dilutions. Express values as gc/mL, and 423 divide by the titer expressed as PFU/mL. Calculate the 424 gc/PFU ratio (*see* **Note 16**). 425

426

3.7 Detection ofThis assay allows the detection of the transgenic protein encoded by 427**Transgene Expression**The recombinant virus. The following protocol refers to an assay in 428SK-OV-3 cell line.429

- 1. Seed a 12-well cell culture plate with 5×10^5 cells in 1 mL 430 RPMI-GlutaMAX/10% FBS Δ (*see* Note 12). Incubate o/n at 431 37 °C in a CO₂ incubator. 432
- 2. Infect the cell monolayers with the recombinant virus expres- 433 sing the transgene (e.g., R-115 engineered to encode mIL12) 434 or with the control recombinant virus (e.g., R-LM113, same 435 backbone, but no transgene) at 0.1–1 PFU/cell in 350 μ L of 436 low serum medium. Incubate at 37 °C for 90 min on a rocking 437 shaker. 438
- Replace the virus inoculum with 1.5 mL of low serum medium. 439 Incubate plates at 37 °C for 3 days in a CO₂ incubator. 440

Follow steps 4 and 5 for detection of transgene expression by 441 ELISA. 442

- 4. At 24, 48, and 72 h postinfection, withdraw an appropriate 443 volume $(150-300 \,\mu\text{L})$ of culture medium from each well, pellet 444 and discard any cell, recover the supernatant and snap freeze in 445 liquid nitrogen to avoid protein degradation. 446
- 5. Proceed with transgenic protein quantification, using a com-447 mercial or in house ELISA kit. Express the concentration of 448 secreted protein as pg/mL. For example, to quantify mIL12 449 secreted by cells infected with R-115 recombinant virus, 450 150 μL of medium are taken from wells infected with R-115 451 or R-LM113 (control), at 24, 48, and 72 h after infection. 452 50 μL of each sample is used in ELISA, in duplicate, following 453 the manufacturer's instructions. To eliminate matrix effect on 454 the values, averages of the replicates of the mIL12-positive 455 recombinant virus R-115 are subtracted of mIL12 background 456 values detected in the medium of the control mIL12-negative 457 recombinant virus (e.g., R-LM113 [4]).

Follow steps 6–10 for detection of transgene expression by 459 flow cytometry. 460

- 6. At 24, 48, and 72 h postinfection, remove the medium and 461 detach cells using a scraper, or by trypsinization. 462
- 7. Pellet cells at $400 \times g$ for 7 min, then resuspend the pellet in 463 50 µL of ice-cold FACS buffer to dissociate any clump. Keep 464 cells on ice for the rest of the experiment. 465

- 8. React cells with the appropriate dilution of fluorochromeconjugated antibody directed against the transgenic product.
 Keep a sample unstained as negative control. Incubate on ice for 30 min (*see* Note 17).
 469
- 9. Wash cells twice with 1 mL of FACS buffer, pelleting at $400 \times g$ 470 for 7 min. Resuspend pellets in 300 µL of FACS buffer. 471
- 10. Acquire the sample data by flow cytometry $(1-5 \times 10^4$ events472in the gate, per sample) with the appropriate filters. Express473the data as the mean intensity of the fluorescence signal of the474stained cells, after subtraction of the mean fluorescence intensity of the cells reacted with the secondary antibody only.476

Follow **steps 11–13** for detection of transgene expression by 477 Western blot. 478

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.

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.

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- 13. Use the same amount of proteins (in the range of $10-250 \mu g$) 489 or the same volume of medium (for secreted proteins) for 490 SDS polyacrylamide gel electrophoresis (SDS PAGE). Trans-491 fer the proteins to a nitrocellulose or PVDF membrane and 492 detect transgenic product and control proteins (e.g., tubulin 493 or β -actin) with appropriate antibodies. Develop WB with 494 ECL reagents, detect and quantify signals as appropriate. 495 For a qualitative assay, compare cell lysates infected by 496 transgene-expressing or -non-transgene-expressing viruses. 497 For a semiquantitative analysis, use known amounts of the 498 purified transgenic product to create a standard curve in the 499 blot. Use the curve to calculate the amount of transgenic 500 product expressed by the infected cells. 501
- 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.
- Use 2 μg of RNA for cDNA synthesis, with a retrotranscription 507 kit, according to the manufacturer's instructions. 508
- 3. Dilute the cDNAs in $ddH_2O(1:5)$ and use 2 μ L in a qRT-PCR 509 reaction. For the quantification of transgenic mIL12 expressed 510

3.8 Detection of Transgene mRNA Expression by qRT-PCR from R-115-infected cells, a qRT-PCR assay is performed, 511 using the probes for mIL12 (Mm00434169_m1) and for a 512 housekeeping gene of SK-OV-3 cells (human gapdh, Taqman 513 assay Hs99999905_m1). Calculate results by means of Δ ct 514 method, comparing the expression of mIL12 in cells infected 515 with R-115- or the control mIL12-negative R-LM113 recombinant virus. 517

518

549

- 1. Seed a 24-well cell culture plate with the cell lines of choice (*see* 519 Note 12). Incubate at $37 \,^{\circ}$ C in a CO₂ incubator. 520
- 2. Infect cells at 2–10 PFU/cell with the recombinant and the wt 521 control virus, or mock-infect. Infections are carried out in 522 200 μ L of low serum medium. Incubate at 37 °C for 90 min 523 on a rocking platform. 524
- 3. Replace the viral inoculum with 500 μ L of fresh low serum 525 medium. Incubate for 24–48 h at 37 °C in a CO₂ incubator. 526
- 4. If the recombinant virus expresses a fluorescent marker, moni-527 tor infection by fluorescence microscopy with the appropriate 528 filters. Otherwise, an immunostaining can be performed (*see* 529 Subheading 3.5, step 5). 530
- To quantitatively measure the infected cells, analyze samples by 531 flow cytometry. After steps 1–3, remove the medium and 532 detach cells using a scraper, or trypsin–EDTA.
- 6. Pellet cells at $400 \times g$ for 7 min, then resuspend the pellet in 534 50 µL of ice-cold FACS buffer to dissociate any clumps. Keep 535 cells on ice for the rest of the experiment. 536
- 7. If recombinant virus expresses a fluorescent marker, go to step 537
 9. Otherwise, select a virus-expressed protein, which is loca-538 lized on the surface of the cell, and use the appropriate amount 539 of fluorochrome-conjugated antibody directed against this 540 protein (*see* Note 17). Incubate on ice for 30 min. 541
- 8. Wash cells two times with 1 mL of FACS buffer, pelleting at 542 $400 \times g$ for 7 min. Resuspend in 300 µL of FACS buffer. 543
- 9. Acquire the sample with a flow cytometer with appropriate 544 filters for the fluorochrome $(1-5 \times 10^4 \text{ events})$ in the gate, per 545 sample). Use the signal of the mock-infected cells to set the 546 "zero" of the fluorescence, and express the infection as the 547 percentage of infected cells. 548

The protocol below refers to an assay carried out in SK-OV-3 cells 550 to measure the kinetic of recombinant virus production in infected 551 cells. 552

> 1. Seed 12-well cell culture plates with 5×10^5 cells in 1 mL 553 RPMI-GlutaMAX/10% FBS Δ (*see* Note 12). Incubate at 554 37 °C in a CO₂ incubator overnight. The number of plates 555

3.9 Extent of Infection

3.10 Extent of Recombinant Virus Replication

corresponds to the time points to be analyzed (usually at least 556 two, for 24 and 48 h). 557 2. Infect cells at 0.1-1 PFU/cell. Infections are carried out in 558 350 µL of low serum medium (RPMI-GlutaMAX/2.5% 559 FBS Δ). Incubate for adsorption and entry at 37 °C for 560 90 min on a rocking shaker. 561 3. To inactivate unpenetrated recombinant virus, wash once with 562 PBS, then perform an acidic wash (pH 3 wash) for 1 min. Wash 563 twice with 1 mL of PBS. Then, add 1 mL/well of low serum 564 medium. Incubate at 37 °C in a CO₂ incubator. 565 4. Block the infections at the chosen time points (24 and 48 h) by 566 freezing the plate at -80 °C. 567 5. Seed 12-well plates for titration (see Subheading 3.5). 568 6. That the frozen plates on ice, scrape the bottom of each well 569 and collect the medium with the cell lysate in 2 mL Eppendorf 570 tubes. 571 7. Sonicate the content of the tubes to release the viral particles 572 from the cells. 573 8. Perform titration with serial dilutions as described (see Sub-574 heading 3.5). Express results as PFU/mL or PFU/cell at 575 24 and 48 h. 576 577 578

4 Notes

tipata 570

- 1. When handling HSV-BAC DNA always use wide-orifice tips to prevent DNA fragmentation.
 579

 580
 580
- 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.
- 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 599 under the fluorescence microscope. 600

- 5. Take aliquots during the purification process for analysis and 601 titration to monitor recovery at every step. 602
- 6. This procedure is devised for the preparation of virions from 603 10 T175 flasks of SK-OV-3 cells. The protocol can be scaled up 604 or down depending on specific needs.
 605
- 7. SK-OV-3 cells never detach completely from the flask, but all 606 cells should be rounded up.
 607

- 8. Higher *g*-force can make virion resuspension difficult.
- 9. This will allow the virion pellet to resuspend more easily. It is 609 pivotal to avoid drying of the pellet during the incubation 610 on ice.
- 10. For the production of recombinant virions, which may repli-612 cate more slowly than wt virus, lowering the temperature to 613 33 °C slows down cell growth allowing more time to recombi-614 nant virus replication and avoiding cells outgrowing the virus. 615 After 5 days, cells will look strongly altered, but will neverthe-616 less give a good virus yield. For every combination of recombi-617 nant virus and host cell line, it is worth comparing the 618 recombinant virus growth in standard conditions (2–3 days at 619 37 °C) with the low temperature conditions (1 day at 37 °C 620 followed by 4 days at 33 °C).
- 11. To speed up the process, you can also first fill the tube with 622 supernatant and afterward add quickly 1.5 mL of 50% iodix- 623 anol WS going at the bottom of the tube.
- 12. Seed the wells with a number of cells suitable to achieve 100% 625 confluency after an overnight incubation. Do not exceed with 626 the number of cells: infection and titration assays performed in 627 highly dense monolayers can lead to underestimation of the 628 actual recombinant virus titer. 629
- 13. According to the expected titer and the quantity of recombinant virus available, serial dilutions may start from 10^{-1} , made 631 by adding 50 µL of recombinant virus to 450 µL of low serum 632 medium. For small amounts of concentrated recombinant 633 virus (usually virions) the first dilution is 10^{-2} , made by adding 634 5 µL of recombinant virus to 495 µL of low serum medium. All 635 the subsequent tenfold dilutions are prepared by adding 50 µL 636 of the previous dilution to 450 µL of low serum medium. 637
- 14. Calculation of the titer: number of plaques 638 (PFU) $\times 10^{(-\text{dilution})}/0.35$ mL. For example, 23 plaques in 639 dilution -8 correspond to 23 PFU $\times 10^8/0.35$ mL $= 6.6 \times 10^9$ 640 PFU/mL. For accuracy and statistical significance, the titrations should be carried out in duplicate or triplicate. 642

- 15. The choice of fixing solution depends on the primary antibody 643 to be used for the immunostaining. For different antigens, the 644 optimal combination of fixing conditions and working anti-645 body concentration must be determined by the operator. 646
- 16. Recombinant virion preparations are typically in the range of 647 200-300 gc/PFU in SK-OV-3, which means one infectious 648 virion every 200-300 virions. This value indicates a great prev-649 alence of nonencapsidated genomes (>99.5%) over the infec-650 tious virions. 651
- 17. As an alternative to a fluorochrome-conjugated antibody 652 directed against the transgene product, it is possible to use a 653 primary antibody directed to the antigen of interest, followed 654 by a fluorochrome-conjugated secondary antibody. The opti-655 mal working antibody concentrations must be determined by 656 the operator. 657

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