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oHSV Genome Editing by Means of galk Recombineering

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

Abstract

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Since the cloning of the herpes simplex virus (HSV) genome as BAC (bacterial artificial chromosome), the 7 genetic engineering of the viral genome has become readily feasible. The advantage is that the modification 8 of the animal virus genome is carried out in bacteria, with no replication or production of viral progeny, and 9 is separated from the reconstitution or regeneration of the recombinant virus in mammalian cells. This 10 allows an easy engineering of essential genes, as well. Many technologies have been developed for herpesvi-11 rus BAC engineering. In our hands the most powerful is *galK* recombineering that exploits a single marker 12 (*galK*) for positive and negative selection and PCR amplicons for seamless modification in the desired 13 genome locus. Here we describe the engineering of the HSV recombinant BAC 115 by the insertion of a 14 heterologous cassette for the expression of murine interleukin 12 (mIL12) in the intergenic sequence 15 between US1 and US2 ORFs. 16

Key words Herpes simplex virus, Oncolytic virotherapy, Virus engineering, *galK* recombineering, 17 Virus arming, Transgene expression, Interleukin 12 18

1 Introduction

OVs (oncolytic viruses) belong to different virus families and, 20 consequently, exhibit different pros and cons. The advantages of 21 herpes simplex virus (HSV) as an oncolytic agent are as follows: 22

- A detailed knowledge of the genomic arrangement and of the ²³ function of the viral gene products. ²⁴
- A large genome (about 150 kbp) which enables the insertion of a 25 number of foreign genes, up to three to four as of now. In 26 principle, this number can be substantially further increased. 27
- The viral genome does not integrate into the host genome. 28
- The technologies for the genetic engineering are well 29 established. 30

- The ability to elicit a strong antiviral and antitumor innate 31 immunity; in essence oHSVs behave as antigen-agnostic anti-32 cancer vaccines. 33
- The ability to synergize with checkpoint blockade, and to confer sensitivity to and widen the activity of checkpoint inhibitors.
 34
- Last but not least, a specific anti-herpes drug is available in a worst-case scenario, a unique advantage of herpesviruses.

The major disadvantage of oHSV is possibly the prior immunity in the human host. This limitation can be in part counteracted by the route of administration. Because of these properties oHSVs have been among the first viruses to be tested as oncolytic agents [1, 2]; *see* also refs. 3, 4. The first registered OV belongs to the herpesviridae family [5–7].

The application of molecular biology techniques to herpes-44 viruses has been critical for development and improvements of 45 technologies aimed at generating HSV recombinants with the 46 desired modifications. Of the various genetic engineering technol-47 ogies that our laboratory has applied over time, we found that *galK* 48 (galactokinase) recombineering (recombination-mediated genetic 49 engineering) is straightforward, relatively easy to design and to 50 carry out with high rate of success. It requires the prior cloning of 51 the herpesviral DNA as BAC (bacterial artificial chromosome) 52 [8, 9]. The insertion of BAC sequences in HSV was described in 53 detail [10-13] and was covered previously in this book series 54 [14]. *galK* recombineering allows the introduction of the planned 55 genetic modification into HSV-BAC DNA in bacteria (E. coli). Of 56 note, even essential viral genes can be readily manipulated, since no 57 viral gene expression takes place in the bacterial host. Following 58 recombineering, recombinant virus can be rescued upon transfec-59 tion of the genetically modified (recombinant) HSV-BAC DNA in 60 mammalian cells, susceptible and permissive to HSV. Of note, 61 several types of viral modifications can be obtained, including 62 point mutations, deletions/insertions, marker or transgene expres-63 sion, changes to viral tropism, etc. 64

The initial technologies for HSV-BAC engineering in E. coli 65 (two-step replacement strategy) involved the RecA-mediated 66 homologous recombination of the HSV-BAC with a transgene 67 carried by a shuttle plasmid and the use of two different markers 68 to select for the cointegrate and the resolved final genome 69 [15]. The technology had some pitfalls, mainly because of low 70 stringency of the marker employed in the counterselection of the 71 unresolved recombination intermediates. In particular, the sacB 72 marker present in the shuttle vector was quite easily inactivated 73 and lost its efficacy. In addition, the cloning of the desired modified 74 viral gene or heterologous transgene in a shuttle vector prevented 75 in most cases a seamless insertion of the desired mutations, due to 76 the restriction sites used for cloning. 77

Strategies involving double crossing-over like lambda (λ) 78 Red-mediated homologous recombination or ET-recombination 79 circumvented the issue of seamless insertions, as they exploit PCR 80 generated linear fragments with short homology arms (50–60 bp) 81 to the target site. The need to express the recombinases from an 82 additional plasmid and only for a short time period required an 83 extra transformation step [16]. These methods were generally 84 employed for gene inactivation or deletions, in combination with 85 the insertion of an antibiotic resistance marker followed by 86 FRT-Flp recombination, for example, as we did to delete HSV 87 glycoprotein D gene [17]. 88

The *galk* recombineering technology has been developed by 89 Warming et al. and proposed as an innovative and powerful tool for 90 the engineering of BAC DNAs [18]. The advantages are twofold. 91 First, the λ Red-mediated homologous recombination is carried 92 out by recombinases transiently and inducibly expressed from the 93 genome of an appropriate *E. coli* host strain, SW102. Second, a 94 single marker, *galK*, is used for positive and negative selection, that 95 is, to target the desired site of insertion, and, subsequently, for the 96 replacement of the *galK* insert with viral sequences carrying the 97 desired modification. This allows seamless modifications of the 98 BAC and avoids the need of transformation of recombinase-99 expressing plasmids. More importantly, it enhances the efficiency 100 of recovery of the final product, by putting a negative selection on 101 the constructs that still carry the *galK* marker. 102

In this chapter we provide an example of the insertion of a 103 transgene in HSV-BAC, by means of *galK* recombineering. The 104 subsequent steps for the rescue and cultivation of the recombinant 105 virus are illustrated in a separate chapter of this book (*see* 106 Chapter 8). We highlight the specific problems that can arise in 107 the procedure. The specific example is the generation of R-115, 108 that is, the insertion of mIL12 in R-LM113, an oHSV retargeted to 109 HER2 cancer receptor and expressing the EGFP reporter gene 110 (Fig. 1).

2 Materials

2.1 HSV-BAC DNA Extraction and Electroporation into E. coli SW102 1. *E. coli* bacterial strain SW102, derived from DH10B strain, is 113 used to harbor and amplify HSV-BACs, and for BAC recombineering using *galK* positive/negative selection [18]. SW102 115 strain must be grown at a temperature not exceeding 32 °C, to 116 avoid unwanted expression of the three λ Red-encoded genes 117 (*exo*, *bet*, and *gam* recombinases) from a stably integrated 118 defective λ prophage (*see* **Note 1**). 119

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Fig. 1 Schematic diagram of *galK* recombineering for the generation of HSV-BAC 115. The line lengths and box sizes are representative and are not drawn to scale. (a) The starting HSV-BAC LM113 carries EGFP as reporter gene (green box). The two halves of the intergenic sequences (IGS) between US1 and US2 are depicted as magenta and red boxes. The PCR amplicon of the *galK* cassette (orange box) carries upstream and downstream arms homologous to the IGS. (b) Following the first step of *galK* recombineering with *galK* positive selection, the *galK* cassette is inserted in the US1-US2 IGS. (c) The pCMV-mIL12 cassette (blue box) is amplified with primers carrying the same homology arms for the IGS (magenta and red boxes). (d) After the second step of *galK* recombineering and *galK* negative selection the pCMV-mIL12 cassette is inserted in place of *galK*, and the final recombinant HSV-BAC 115 is generated. *EGFP* enhanced green fluorescence protein, *IR* inverted repeats, *UL* unique long, *US* unique short

- E. coli containing HSV-BAC DNA (e.g., DH10B containing HSV-BAC LM113 [17]).
 121
- 3. "Low salt" LB medium (*see* Note 2): 10 g Bacto tryptone, 5 g $_{122}$ yeast extract, and 5 g NaCl in 1 L ddH₂O. Autoclave for $_{123}$ 20 min at 121 °C. $_{124}$
- 4. Chloramphenicol, 20 mg/mL in EtOH.
- 5. BAC DNA extraction kit (e.g., NucleoBond[®] BAC 100 kit 126 (Macherey-Nagel), containing buffers S1, S2, S3, N3, and 127 N5).

125

- 6. STE Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 129 0.1 M NaCl. 130
- 7. Isopropanol.
- 8. 70% ethanol. 132

2.2 Buffers and Solutions for galK **Recombineering and** Characterization of Clones

- 1. $10 \times$ M9 medium: 60 g Na₂HPO₄, 30 g KH₂PO₄, 10 g 134 NH₄Cl, 5 g NaCl in 1 L ddH₂O, autoclave. 135
- 2. 5× M63 buffer: 10 g (NH₄)₂SO₄, 68 g KH₂PO₄, 2.5 mg 136 FeSO₄·7H₂O in 1 L ddH₂O, adjust to pH 7.0 with 10 N 137 KOH, autoclave. 138
- 3. Supplements for M63 minimal plates: D-biotin (0.2 mg/mL, 139 sterile filtered); D-galactose (20%, autoclaved); 2-deoxy-galac- 140 tose (DOG, 20%, prepared just before use, sterile filtered, see 141 Note 3); glycerol (20%, autoclaved); L-leucine (10 mg/mL, 142 dissolved by heating, then cooled down and sterile filtered); 143 MgSO₄·7H₂O (1 M, autoclaved); Chloramphenicol (20 mg/ 144 mL in EtOH). 145
- 4. M63 minimal plates: autoclave 15 g Bacto agar (see Note 4) in 146 800 mL of ddH₂O, cool down to 50 °C; add 200 mL of $5\times$ 147 M63 medium and 1 mL of 1 M MgSO₄·7H₂O; adjust volume 148 to 1 L with sterile ddH_2O if necessary. Add 5 mL biotin (1 mg), 149 4.5 mL leucine (45 mg), and 625 µL chloramphenicol 150 (12.5 µg/mL working concentration). Complete with the car-151 bon source and/or selective compound: for galactose minimal 152 plates, add 10 mL of 20% D-galactose; for DOG minimal plates, 153 add 10 mL of 20% glycerol and 10 mL of 20% 2-deoxy-154 galactose. 155
- 5. MacConkey indicator plates: prepare MacConkey agar accord- 156 ing to the manufacturer's instructions (see Note 5), autoclave, 157 cool at 50 °C, add D-galactose (1% final concentration) and 158 chloramphenicol (12.5 μ g/mL working concentration). 159
- 6. High fidelity polymerase and buffer (e.g., Phusion Polymerase 160 (Thermo Fisher)). 161
- 7. Taq polymerase and buffer. 162
- 8. 50 mM MgCl₂. 163 9. dNTP mix, 10 mM. 164
- 10. Restriction endonucleases *DpnI*, *BamHI*, *Eco*RV, and *KpnI*. 165 11. 1-kb DNA ladder.

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- 12. SK-OV-3, human ovarian cancer cell line (ATCC HTB-77). 167
- 169 2.3 Nucleic Acids 1. pgalK for *galK* knockin [18] (Fig. 2a). 170 2. pLM84, template plasmid for PCR amplification of a pCMV-171 mIL12 expression cassette for recombination in HSV-BAC 172 [19] (Fig. 2b). 173 174 2.4 Agarose Gel 1. 50× TAE buffer (1 L): 242 g of Tris base, 57.1 mL of acetic 175**Electrophoresis Buffer** acid (glacial), 100 mL of 0.5 M EDTA (pH 8.0). Working 176

13. Lipofectamine 2000.

dilution: $0.5 \times$.



Fig. 2 Maps of the plasmids used for *galK* recombineering. (**a**) pgalK is the template to amplify the *galK* cassette. The positions where chimeric primers anneal are depicted as purple arrows. (**b**) pLM84 is used as template for the amplification of the pCMV-mIL12 cassette [19]. mIL12b (subunit b) and mIL12a (subunit a) coding sequences (blue arrows) are separated by an IRES (yellow). The cassette is under control of CMV promoter and ends with the BGH poly (A) site. Graphics were created with SnapGene

2. Ethidium bromide.	178
3. Agarose.	179
	180
1. Bench instrumentation: benchtop centrifuges, spectrophotom-	181
eter, electroporation system and electroporation cuvettes	182
(0.2 cm), Speed-Vac.	183

2.5 Equipment

193

2. Molecular biology: PCR thermal cyclers, gel electrophoresis system (for short and long run lengths), gel imaging system.	184 185
3. Incubators and shakers for bacteria.	186
4. Folded filter paper.	187
5. Ice-water slurry.	188
6. Eppendorf tubes.	189
7. 50 mL conical tubes.	190
8. 500 mL bottles.	191
	192

3 Methods

This collection of protocols describes the methods to modify an 194 HSV-BAC by *galK* recombineering technologies. In the next chap-195 ter, we will describe how to rescue the recombinant HSV, and 196 finally evaluate its biological properties in vitro. We focus here on 197 the expression of a heterologous gene, the cytokine murine inter-198 leukin 12 (mIL12) in a replication-competent fully retargeted 199 HSV, by insertion of an expression cassette in the non-coding 200 intergenic region between US1 and US2 genes (Fig. 1).

3.1 HSV-BAC DNA Extraction

In order to manipulate and engineer the HSV-BAC DNA of interest, a critical feature is the *E. coli* strain. The *galK* recombineering 203 procedure requires SW102 cells, a strain derived from DH10B, 204 engineered with the λ prophage encoding the recombinases, and 205 deleted of the galactokinase gene (*galK*) in the galactose operon 206 [18]. If the HSV-BAC is hosted in a different bacterial strain, its 207 DNA must be extracted and transferred by electroporation into 208 SW102 bacteria. 209

In the example described in this chapter, the starting material is 210 HSV-BAC LM113 in DH10B cells [17]. For the extraction of 211 HSV-BAC DNA we use NucleoBond[®] BAC 100 kit (Macherey- 212 Nagel) (*see* Note 6). Throughout the protocol, maximum care 213 should be taken to avoid shearing of the high-molecular weight 214 HSV-BAC DNA. 215

- Cultivate bacterial cells overnight at 30 °C in 200 mL LB 216 medium + antibiotics (12.5 μg/mL chloramphenicol) (see 217 Note 7).
- 2. Harvest bacteria by centrifugation at $3000 \times g$ for 15 min at 219 4 °C; resuspend the pellet in STE buffer and centrifuge the 220 bacteria again (*see* **Note 8**). Discard supernatant. 221
- 3. Thoroughly resuspend the pellet in 12 mL resuspension buffer 222 (S1) supplemented with 100 µg/mL RNase A. 223
- 4. Add 12 mL of room-temperature lysis buffer (S2) to the sus- 224 pension, mix gently by inverting the tube 6–8 times; the 225

solution turns viscous. Incubate at room temperature 226 (18–25 °C) for max 5 min (*see* Note 9). 227

- 5. Add 12 mL pre-cooled ice-cold neutralization buffer (S3) to the suspension. Promptly mix the lysate gently by inverting the tubes 6–8 times (*see* Note 9). A non-viscous suspension of off-white flocculate must form. Incubate the suspension on ice for 5 min.
- 6. Equilibrate a NucleoBond[®] BAC 100 (anion-exchange resin) 233 column with 2.5 mL of equilibration buffer (N2). Allow the column to completely empty by gravity flow and discard flow-through. 236
- 7. Clarify the lysate through a wet folded filter paper placed in a funnel (*see* Note 10); collect the flow-through.
 238
- 8. Load the cleared lysate onto the equilibrated NucleoBond[®] 239 BAC 100 column; allow the column to empty by gravity flow, 240 discard flow-through. 241
- 9. Wash the column twice with 12 mL wash buffer (N3) and 242 discard flow-through. 243
- 10. Elute the BAC DNA with 5 mL elution buffer (N5) preheated
 at 50 °C (see Note 11); Gently mix and divide in five 1 mL
 aliquots (see Note 12). To avoid shearing of HSV-BAC DNA,
 use wide-orifice tips.
- 11. To precipitate the eluted HSV-BAC DNA add 0.7 mL of room-temperature isopropanol to each aliquot. Mix carefully by inversion (do not vortex) and centrifuge at 14,000 × g for 250

30 min at 4 °C. Carefully remove and discard the supernatant. 251

- 12. Add 1 mL ice-cold 70% ethanol to the pellet of each aliquot,
centrifuge at 14,000 \times g for 10 min at 4 °C. Speed-Vac dry for
10 min.252
253
- 13. Dissolve each aliquot of HSV-BAC DNA in 10 μ L of sterile 255 ddH₂O. Let the pellet hydrate for about 15 min. To avoid 256 shearing of HSV-BAC DNA, pipet gently using wide-257 orifice tips. 258
- 14. Determine DNA yield using an UV spectrophotometer and check HSV-BAC DNA integrity by agarose gel electrophoresis (Fig. 3). Store the purified DNA at 4 °C (*see* Note 13).
 261
- Pick a single colony of SW102 containing no BAC from a plate, and make a 5 mL overnight culture at 30 °C (*see* Note 14).
- 2. Dilute the overnight culture 1:50 in 100 mL of low salt LB 265 medium (*see* Note 15), without antibiotics, in a bottle or a 266 flask. Measure bacterial density (OD_{600}) at time 0 (it should be 267 at least 0.03). Incubate the culture for about 3–4 h in a shaker 268 at 30 °C (in an incubator or in a water bath) and measure 269 bacterial density at regular time intervals. 270

3.2 Electroporation of HSV-BAC into SW102 Bacterial Strain



Fig. 3 Typical band pattern of an intact HSV-BAC DNA run on a 0.8% agarose gel in $0.5 \times$ TAE. The bands are sharp, without smear. MW: GeneRuler 1 kb DNA Ladder; sizes are in base pairs (bp). M: MassRuler DNA Ladder Mix (Thermo Scientific): *: 50 ng, **: 40 ng

- During the bacterial growth, prepare an ice–water slurry and 271 cool on ice 1 L of sterile ddH₂O, 50 mL conical tubes, Eppen-272 dorf tubes, 0.2 cm electroporation cuvettes (*see* Note 16). 273
- 4. When the OD_{600} of the culture is near 0.6, the bottle contain- 274 ing the bacteria is cooled down in the ice-water slurry for 275 1–2 min, and subsequently transferred into precooled 50 mL 276 conical tubes. 277
- 5. Spin down the bacteria in a cold (0–1 °C) centrifuge for 8 min 278 at $3000 \times g$. 279
- 6. Pour off all the supernatant, then add 5 mL ice-cold ddH₂O, 280 while keeping the tube with the bacterial pellet in the ice-water 281 slurry. Resuspend the pellet in water by gently shaking the tube 282 in the ice-water slurry (the first time it will take about 5 min). 283 When resuspended, fill up to 50 mL with ice-cold ddH₂O, mix 284 by inversion, and spin in a cold centrifuge for 8 min at 285 $3000 \times g$. 286
- 7. Make a second cold water wash as in **step 6**, then remove all ²⁸⁷ supernatant by inverting the tube on a paper towel. Gently ²⁸⁸ resuspend the bacterial pellet in the residual small amount of ²⁸⁹ ddH_2O left in the tube and store the competent cells on ice. ²⁹⁰
- Transfer 50 μL of the freshly made electrocompetent cells to a 291 pre-cooled Eppendorf tube and mix with 100 ng–1 μg of the 292

HSV-BAC DNA to be transformed. Transfer to a precooled 293 0.2 cm electroporation cuvette. 294

- 9. Transform by electroporation (200 Ω , 25 μ F, 2.5 kV), and immediately add 1 mL of ice-cold low salt LB medium to the cuvette. Transfer bacteria to an Eppendorf tube and incubate in a shaker at 30 °C for about 1 h. 298
- 10. Plate different amounts (1, 10, or 100 μ L, and all the rest) of299the transformed bacteria on low salt LB agar plates plus chlor-300amphenicol (12.5 μ g/mL) to obtain single colonies (*see* Note30117). Incubate at 30 °C for 1–2 days.302

303

3.3 Galk The *galK* recombineering technology allows to modify a BAC 304 DNA cloned in *Escherichia coli* via lambda (λ) Red-mediated 305 Recombineering homologous recombination. In this strategy, the use of restriction 306 enzymes and DNA ligases to modify DNA is not required, instead 307 BAC DNA is modified using *galK* positive/negative selection in a 308 two-step procedure [18]. The first step is a homologous recombi-309 nation to insert (knockin) the *galK* cassette into the desired posi-310 tion of the HSV-BAC DNA. Recombinant clones are selected by 311 positive selection of bacteria that acquired the ability to grow on 312 minimal media with galactose as the only carbon source. The 313 second step involves another homologous recombination to sub- 314 stitute the galK cassette (galK knockout) with the sequence of 315 interest. In this case a negative selection against galK with 316 2-deoxy-galactose (DOG), toxic following phosphorylation by 317 the galK gene product, ensures the identification of recombinant 318 clones. Both recombinations occur via short homology sequences 319 or homology arms, which flank the *galK* or the custom cassette, 320 and are homologous to the selected target position in the 321 HSV-BAC DNA (Fig. 1). 322 323 3.3.1 GalK Knockin The first step of *galK* recombineering technology consists of the 324 (Positive Selection) insertion of the constitutively expressed galactokinase cassette 325 (galK) into the HSV-BAC locus chosen for the selected modifica-326 tion. To this purpose, first, it is necessary to PCR amplify the galK 327 cassette flanked by short homology arms included in the primers 328 (see Note 18). 329

1. Design PCR chimeric primers for the amplification of the galk 330 cassette. For the insertion described here, we used the follow-331 ing primers: US1/US2_galK_f ATAAAAGACCAAAAT- 332 CAAAGCGTTTGTCCCAGCGTCTTAATGGCGG-333 GAAGCCTGTTGACAATTAATCATCGGCA, and 334 US1/US2 galK r AATAAACCCCCAAACACCCCC- 335 CATGTACGCGTGGTCTGTTTCTCTCCGCCTCAG-336 CACTGTCCTGCTCCTT (arms with homology to 337 HSV-BAC are in *italics*, whereas the sequences annealing to 338 *galK* cassette are in plain text). 339

- 2. Set up the PCR reaction with a high fidelity polymerase (here a 340 50 μ L reaction with ThermoFisher Phusion polymerase is 341 reported): 10 μ L of 5× Phusion HF buffer, 1 μ L of 10 mM 342 dNTPs, 2.5 μ L of 10 μ M forward and reverse primers (0.5 μ M 343 final concentration), 0.5 μ L of Phusion polymerase; 33 μ L of 344 ddH₂O. Finally, add 1 μ L of 2 ng/ μ L pgalK plasmid as tem- 345 plate. Amplification conditions: initial denaturation and hot 346 start at 98 °C for 30 s, then 32 cycles at 98 °C for 10 s, 347 58 °C for 20 s, 72 °C for 30 s, final extension at 72 °C for 348 10 min. Run 5 μ L of the PCR product on a 0.8% agarose gel. A 349 band of ~1.3 kbp is expected.
- 3. Digest the PCR product with 2 μ L (40 U) of DpnI restriction 351 enzyme/50 μ L reaction, for 1 h at 37 °C to remove the 352 methylated template. 353
- 4. Run the DpnI digestion on a 0.6% agarose gel (1 h at 80 V) and 354 purify the *galK* band by gel extraction spin columns. Elute the 355 *galK* fragment in 50 μ L nuclease free ddH₂O (*see* **Note 19**). 356
- 5. Measure the concentration of the *galK* fragment with an UV $_{357}$ spectrophotometer and dilute it to a final concentration of $_{358}$ 30 ng/µL with ddH₂O. $_{359}$
- 6. Recombineering for *galK* positive selection. Inoculate a single 360 bacterial colony containing the recipient HSV-BAC (in this 361 example BAC LM113) in 5 mL of low salt LB + 12.5 μ g/mL 362 chloramphenicol and incubate with shaking at 30 °C 363 overnight. 364
- 7. Dilute 2 mL of the overnight culture in 100 mL of low salt 365 LB + 12.5 μ g/mL chloramphenicol and shake it at 30 °C to 366 reach an OD₆₀₀ between 0.55 and 0.65. This step takes about 367 3.5 h. In the meantime precool in an ice–water slurry 1 L of 368 sterile ddH₂O, 50 mL conical tubes, Eppendorf tubes, 0.2 cm 369 electroporation cuvettes. 370
- 8. Divide the culture in two 500 mL bottles. Shake one bottle in a 371 42 °C water bath for 15 min (*see* **Note 20**) to induce λ pro- 372 phage recombinases (induced sample), leave the other bottle at 373 30 °C (uninduced control). 374
- 9. Cool the two cultures (hereafter designated as "induced" and 375 "uninduced") on ice for 5 min, transfer them to 50 mL pre- 376 cooled tubes and centrifuge for 8 min at 3500 × g at 0 °C.
- Pour off all the supernatant, add 5 mL of sterile ice-cold 378 ddH₂O and resuspend the pellet, with gentle swirling in an 379 ice-water slurry (*see* Note 21). Fill the Falcon tubes up to 380 50 mL with sterile ice-cold ddH₂O and pellet again. 381
- 11. Repeat step 10.

- 12. Invert the tubes on a towel to remove the supernatant $_{383}$ completely and gently resuspend the bacterial pellet in the $_{384}$ residual small amount of ddH₂O left in the tube by swirling $_{385}$ in the ice–water slurry. Keep the competent cells on ice. $_{386}$
- 13. Mix 50 μL of cells and 1 μL of *galK* fragment (30 ng/μL) in a precooled Eppendorf tube.
 388
- 14. Transfer and electroporate the DNA-cell mix in a precooled $_{389}$ 0.2 cm electroporation cuvette at 25 µF, 2.5 kV and 200 Ω (*see* 390 **Note 22**). 391
- 15. Add 1 mL of ice-cold low salt LB medium and recover bacteria 392 for 90 min at 30 °C in a shaker. 393
- 16. Wash bacteria (induced and uninduced) with $1 \times M9$ salts (*see* 394 Note 23): pellet the culture (~1 mL) for 15 s at 14,000 × g and 395 remove supernatant carefully with a micropipette, resuspend 396 pellet with 1 mL of $1 \times M9$ salts. 397
- 17. Repeat step 16.
- 18. Plate different amounts (1, 10 or 100 μ L, and all the rest) of 399 the cultures on M63-D-Galactose minimal plates (*see* Note 24). 400

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401

- 19. Incubate plates at 30 °C for 3–4 days (*see* Note 25).
- 20. For screening of recombinants after selection on M63-D-galactose minimal plates, choose ten single colonies from induced sample plates and streak each on a MacConkey indicator plate to make a dilution grid (Fig. 4) (*see* Note 26). Incubate the plates at 30 °C overnight.



Fig. 4 Dilution of bacteria with the grid method. Pick a single colony with a sterile tip, and deposit the excess on the side of the plate by a forward-and-back streak (black doodle #1). With the same tip make a linear streak as in black arrow #2. Change tip and make linear streaks as depicted by the red arrows #3–7. Change again tip and make linear streaks as shown by the green arrows #8–11. This will result in a dilution of bacteria and should produce single colonies in the area highlighted in yellow

- 21. Choose one single brilliant red colony from each MacConkey 407 plate. Repeat the plating with the grid dilution method. This 408 ensures the isolation of pure, non-mixed, clones. Incubate at 409 30 °C overnight.
- 22. Choose one or two single brilliant red colonies from each 411 MacConkey plate and make a small linear streak on low salt 412 LB plates + 12.5 μ g/mL chloramphenicol. Incubate at 30 °C 413 overnight. 414
- 23. Set up a colony PCR reaction to verify the presence of *galK* 415 cassette in the selected position in the HSV-BAC genome. In 416 the example of this chapter we used a forward primer 417 (ACACGTTTCTCCGGCCGTGAGTCCG) 418 US1 1802 f annealing on HSV US1 genomic sequence, and galK_417_r 419 (CATTGCCGCTGATCACCATGTCCACGC) a reverse 420 primer annealing on *galK*, yielding an amplification product 421 of 547 bp. Alternatively, for general purpose galK screening, 422 primers both annealing on *galK* sequences can be used (e.g., 423 (GCGTGATGTCACCATTGAAG) galK 827 f and 424 galK_1142_r (TATTGTTCAGCGACAGCTTG)), yielding a 425 315-bp band (Fig. 5a). 426

Taq protocol (20 μ L reaction): 2 μ L of 10× Taq Buffer, 427 0.6 μ L of 50 mM MgCl₂, 0.4 μ L of 10 mM dNTPs, 1 μ L of 428 10 μ M forward and reverse primers (0.5 μ M final concentration), 0.08 μ L of Taq polymerase, 14.9 μ L of ddH₂O. Finally, 430 as template, pick a tiny amount of bacterial colony, and dissolve 431 it directly in the PCR mix (*see* **Note 27**). When using primers 432 annealing both on *galK*, use 1 μ L of 2 ng/ μ L pgalK plasmid, 433 or a pgalK colony, as positive control. Amplification conditions: initial denaturation at 96 °C for 3 min, then 32 cycles 435 at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s, final extension 436 at 72 °C for 10 min. 437

- 24. Extract HSV-BAC DNA from four positive clones (*see* Sub- 438 heading 3.1) and transfect SK-OV-3 (susceptible and permis- 439 sive) cells with Lipofectamine 2000 (*see* next Chapter, 440 Subheading 3.1) to verify HSV-BAC genome integrity in 441 terms of ability to form plaques, spread and replicate. Monitor 442 the transfected cultures for 3 days for the formation of viral 443 plaques. In our example, plaques are EGFP positive and can be 444 visualized with an inverted fluorescence microscope. 445
- 25. Highly recommended: to check the insertion at the intended 446 position, PCR amplify the *galK* cassette including upstream 447 and downstream flanking regions from HSV-BAC genome, 448 and determine DNA sequence. 449



Fig. 5 Colony PCR pattern for *galK* or heterologous mIL12 cassette. Where a plasmid is used as template for the positive control ("pgalK" or "pLM84") the bands are clean and sharp. Where colonies are used as template, below the specific bands a nonspecific halo is visible, due to the dirty input of bacterial cells. (a) Primers anneal both on *galK* and yield an amplicon of 315 bp. (b) Primers anneal on mIL12b and on IRES and give rise to an amplicon of 300 bp. 1, 2: negative clones; 3: positive clone. MW: GeneRuler 1 kb DNA Ladder. Sizes are in base pairs (bp)

3.3.2 GalK Knockout	The second step of <i>galK</i> recombineering technology consists in the	451
(Negative Selection)	insertion of the transgene (in this example mIL12) in place of <i>galK</i>	452
	cassette in the HSV-BAC clone obtained with the first step of	453
	recombination. The cassette with the transgene of interest is ampli-	454
	fied by PCR including in the primers short arms with homology to	455
	the target the insertions site (Fig. 1).	456
	1. Design PCR chimeric primers for the amplification of the	457
	transgene cassette. For insertion of mIL12, we used the fol-	458
	lowing primers: US1/US2_CMV_f	459
	ATGTCCCCAAATAAAAGACCAAAAT-	460
	CAAAGCGTTTGTCCCAGCGTCTTAATGGCGG-	461
	GAAGCGTTTTGCGCTGCTTCGCGATGTACGGGC, and	462
	US1/US2_polyA_rev	463
	CCCCGATGTCAATAAACCCCCCAAACACCCCC-	464
	CATGTACGCGTGGTCTGTTTCTCTCCGCCGCCATA-	465
	GAGCCCACCGCATCCCCAGCATGCCTG (arms with	466
	homology to HSV-BAC are in <i>italics</i> , whereas the sequence	467

that recognizes pCMV and polyA of mIL12 expression cassette 468 on pLM84 are in plain text). 469

- 2. Set up the PCR reaction with a high fidelity polymerase (here a 470 50 μ L reaction with ThermoFisher Phusion polymerase is 471 reported): 10 μ L of 5× Phusion HF buffer, 1 μ L of 10 mM 472 dNTPs, 2.5 μ L of 10 μ M forward and reverse primers (0.5 μ M 473 final concentration), 0.5 μ L of Phusion polymerase, 33 μ L of 474 ddH₂O. Finally, add 1 μ L of 2 ng/ μ L pLM84 plasmid as 475 template. Amplification conditions are: initial denaturation 476 and hot start at 98 °C for 30 s, then 32 cycles at 98 °C for 477 10 s, 60 °C for 40 s, 72 °C for 4 min. Check the PCR product 478 on a 0.8% agarose gel.
- 3. Digest the PCR product with 2 μ L (40 U) of DpnI restriction 480 enzyme/50 μ L reaction, for 1 h at 37 °C to remove the 481 methylated template. 482
- 4. Run the DpnI digestion on a 0.6% agarose gel (1 h at 80 V) and 483 purify the band of the heterologous cassette by gel extraction 484 spin columns. Elute the fragment in 50 μ L nuclease free 485 ddH₂O (*see* Note 19). 486
- 5. Quantify the transgene fragment with an UV spectrophotome- 487 ter and dilute it to a final concentration of 200 ng/µL with 488 ddH₂O. 489
- 6. For recombineering and *galK* negative selection, inoculate one 490 *galK* positive clone in 3 mL of low salt LB + 12.5 μg/mL 491 chloramphenicol (in this example, BAC LM113 with *galK* 492 inserted at US1–US2 intergenic region).
- 7. Prepare electrocompetent cells as in Subheading 3.3.1, steps 494 7–12. 495
- 8. Mix 50 μL of cells and 1 μL of transgene fragment (200 ng/μ 496 L) in a precooled Eppendorf tube (*see* Note 28).
 497
- 9. Transfer and electroporate the DNA-cell mix in a precooled 498 0.2 cm electroporation cuvette at 25 μ F, 2.5 kV and 200 Ω (*see* 499 Note 22). 500
- Add immediately 1 mL of ice-cold low salt LB, then transfer to 501 a tube containing 9 mL of low salt LB at room-temperature. 502 Recover bacteria for 4.5 h at 30 °C in a shaker (*see* Note 29). 503
- 11. Pellet 1 mL of culture and wash twice in $1 \times M9$ salts as in 504 Subheading 3.3.1, steps 16 and 17. 505
- 12. Plate different amounts (1, 10 or 100 μ L, and all the rest) of 506 the induced and uninduced cultures on M63 DOG minimal 507 plates for selection *against galK* (*see* **Note 24**). 508
- 13. Incubate at 30 °C for 5–7 days (*see* **Note 30**). 509
- 14. Screening of recombinant HSV-BAC DNAs after selection on 510 M63-DOG plates. Choose 30 single colonies from induced 511

sample plates and dilute each on a MacConkey plate indicator 512 plate with the grid scheme (Fig. 4) (*see* **Note 26**). Incubate the 513 plates at 30 °C overnight. 514

- 15. Choose one single white/colorless colony from each MacConkey plate. Repeat the grid dilution on a fresh MacConkey plate.
 This ensures the isolation of pure, not mixed clones. Incubate
 at 30 °C overnight.
 518
- 16. Choose one or two single white/colorless colonies from each 519 MacConkey plate and make a small linear streak on low salt LB 520 plates + 12.5 μg/mL chloramphenicol. Incubate at 30 °C 521 overnight. 522
- 17. Set up two colony PCR reactions to verify the presence of the 523 transgene cassette in the expected position in HSV-BAC 524 genome and the absence of *galK* cassette. In our example we 525 mIL12a 601 f (CATCCTGCTTCACGCCTTCAGused 526 CACCC) and US2_short_r (AACCCCACCCAGCTACCC-527 CAGGCC) for the presence of mIL12 (expected fragment 528 length: 607 bp), and US1_1802_f and galK_417_r (expected 529 fragment length: 547 bp) to verify the absence of galK. Gen-530 eral purpose primers for the mIL12 heterologous cassette are 531 mIL12b_937_f (CAAAGGCGGGAATGTCTGCGTGC) and 532 IRES 201 r (GGGTTCCGCTGCCTGCAAAGGGTCG) 533 (Fig. 5b). 534
- 18. Extract the HSV-BAC DNA from four verified clones (*see* 535 Subheading 3.1), positive for the transgene cassette and negative for *galK*, and transfect them with Lipofectamine 2000 (*see* 537 next Chapter, Subheading 3.1) in the appropriate susceptible 538AU1 and permissive cell line (in this example SK-OV-3). Monitor 539 for the formation of plaques for 3 days. 540

541 542

19. Sequence the transgene cassette from the final HSV-BAC.

3.4 Characterization of Clones Restriction analysis of HSV-BAC clones is a rapid method to differentiate clones with the correct insertion from the aberrant ones. 544 After restriction digestion, for each construct a peculiar restriction 545 enzyme fragments pattern is expected. If possible, include the 546 parental HSV-BAC as control. 547

- 1. Digest 2 μ g of HSV-BAC DNA, extracted from bacteria, with 548 three different restriction enzymes (e.g., 50 U of BamHI, 50 U 549 of EcoRV, 25 U of KpnI) for 6 h or overnight at 37 °C in a total 550 reaction volume of 50 μ L. 551
- 2. Load on a 0.6% agarose gel prepared in $0.5 \times$ TAE and containing ethidium bromide. The gel should have a long run length 553 to allow a good separation of the bands. Include in the same gel 554 a DNA molecular weight marker (e.g., 1 kb DNA ladder). 555



Fig. 6 Check of HSV-BAC 115 DNA integrity by restriction endonuclease analysis with BamHI (B), KpnI (K), and EcoRV (E). Bands were separated on a 0.6% agarose gel in $0.5 \times$ TAE. MW: GeneRuler 1 kb DNA Ladder. Sizes are in base pairs (bp)

- 3. Carry out electrophoresis in $0.5 \times$ TAE buffer at 40 V overnight 556 at room temperature. 557
- 4. The next day, shift the voltage to 60 V for 3–4 h and finally 558 acquire an image with a gel imaging system (Fig. 6). 559

560

- 1. To be on the safe side, we set the incubator and shaking water 562 bath at 29–30 °C. 563
- "Low salt" LB is recommended to prepare electrocompetent 564 SW102 *E. coli*, but we found it suitable for the normal routine 565 propagation of the strain as well. 566

 Prepare just the required amount of 20% 2-deoxy-galactose, to avoid oxidation of the solution. Autoclaving is possible, but not recommended. 	67 68 69
 4. It is critical to use reliable Agar devoid of any carbon source, in order to perform a stringent selection for <i>galK</i>-positive or <i>galK</i>-negative clones. 	70 71 72
 5. Again, it is pivotal to use MacConkey Agar Base totally devoid 57 of lactose or any other carbon source whatsoever, in order to accurately differentiate <i>galK</i>-positive and <i>galK</i>-negative 57 clones. 57 	73 74 75 76
6. NucleoBond [®] PC 100 KIT is suitable for HSV-BAC DNA 57 preparation, as well. 57	77 78
 7. To achieve the highest bacterial growth, perform a small scale culture from a single colony in 5 mL low salt LB + antibiotics for 4–6 h at 30 °C, then dilute it 1:100 for the overnight culture. 	79 30 81 82
8. The STE wash is recommended to remove any trace of culture medium and improve the purity of the extracted HSV-BAC. 58	33 84
9. Do not vortex, to avoid contamination by bacterial chromosomal DNA released from cellular debris. 58	35 86
10. This step is crucial to avoid clogging of the column in the 58 next step. 58	37 88
11 Preheating highly improves elution and recovery of 58	89

HSV-BAC DNA. 590

- 12. This helps in the subsequent steps of precipitation, washing, 591 drying and resuspension. 592
- 13. Avoid freezing at −20 °C: freeze-thaw cycles fragment 593 HSV-BAC DNA. 594
- 14. All the procedures of this protocol must be performed with 595 standard aseptic technique close to a Bunsen burner. 596
- To increase electroporation efficiency, it is important to reduce 597
 NaCl traces in the final suspension of bacterial cells to be electroporated. 599
- 16. Handling bacteria at low temperature increases the efficiency of electroporation. It is important to work quickly on ice during all the next steps of the procedure. An ice–water slurry is preferred to ice only, because the latter harbors air between the ice crystals.
- 17. To make sure to obtain colonies, we advise to plate the rest of $_{605}$ the transformed culture (the pellet from about 890 μ L), too. $_{606}$
- 18. Typical homology arm length is 50 bp. In case of the insertion 607 of large fragments (3 kb or more) or to increase the recombineering efficiency, it can be extended up to 70 bp for 609

synthesized oligonucleotides. Arms longer than 70 bp can be 610 obtained by extending the first PCR product with an extra 611 round of amplification with primers annealing more externally. 612 In a further case, longer homology arms (400 bp or more, 613 upstream and downstream the transgene expression cassette) 614 can be added with traditional cloning. 615

- 19. Use only ddH_2O . Do not use salt-containing buffers, to avoid 616 interference with subsequent electroporation. 617
- 20. Check carefully the temperature of the shaking water bath, 618 possibly with an additional thermometer: a lower temperature 619 may reduce the efficiency of induction, and result in a drastically lower frequency of recombination.
- Resuspension may take a while (up to 10 min). However, from 622 now until the electroporation step, it is very important not to 623 pipet the pellet in order to resuspend bacteria. 624
- 22. Check the output time constant on the electroporation device: 625 good values fall in the range 4.80–4.90 ms. Salt traces in the 626 electrocompetent bacteria suspension or in the fragment will 627 cause a small explosion ("arcing"). If this happens repeatedly 628 with a given batch, it is necessary to prepare new reagents and 629 materials.
- 23. This washing step is essential to remove any rich component of 631
 LB medium from the samples, before the selection on minimal 632
 galactose or DOG plates. It is therefore important to remove 633
 carefully any residual LB medium by means of a micropipette 634
 after every centrifugation. 635
- 24. The number of colonies may vary largely, depending on the 636 quality of the electrocompetent cells and the efficiency of elec-637 troporation and of recombination. Therefore we advise to plate 638 dilutions and all the transformed culture to make sure to obtain 639 single colonies. The 1 and 10 μ L aliquots should be made up 640 with $1 \times M9$ salts to a suggested plating volume of 50–100 µL. 641 The rest of the transformed culture (about 890 μ L) should be 642 pelleted by a short spin in a microfuge (15 s at 14,000 $\times g$), 643 resuspended in 50–100 μ L 1 \times M9 salts and plated. Note that 644 the uninduced control shows a high level of bacterial lysis, 645 visible as viscosity in the sample. 646
- 25. A typical first step of *galK* recombineering (*galK* knockin) 647 yields about 20–30 colonies/ μ L of culture in the induced 648 sample. Usually all of them are positive in *galK* colony PCR. 649 The plates with the uninduced sample should have no colony: 650 the positive selection step is very stringent and uninduced 651 bacteria (in which λ Red recombinases have not been expressed 652 and *galK* cassette has not been inserted into the HSV-BAC) are 653 not able to grow on minimal plates containing D-galactose as 654 the sole carbon source. 655

- 26. Pay attention to streak the excess of colony on the tip on a side 656 of the plate, or you will not obtain single colonies. 657
- 27. Do not exceed with the quantity of input bacterial colony or 658 the PCR reaction may be inhibited. It is best to spread the 659 bacteria first on the wall of the tube, then to push them into the 660 PCR mix, to avoid lumping and sticking of bacteria on the tip. 661 Alternatively, dilute a colony in 20 μ L of ddH₂O, boil for 5 min 662 and use 2 μ L as template for the colony PCR reaction. 663
- 28. In case of a large transgene cassette, it may be useful to perform 664 electroporation with up to 400 ng of PCR fragment. 665
- 29. In the second step of *galK* recombineering the recovery time 666 and the volume are increased with respect to the first step. 667
- 30. Small bacterial colonies will appear for both induced and unin-668 duced sample. This happens because the negative selection 669 (galK counterselection) step is less stringent than the first 670 positive selection step. For this reason a large number of colo-671 nies (sometimes 100+) need to be screened to find recombi-672 nant clones. 673

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