oHSV Genome Editing by Means of galK Recombineering

oHSV Genome Editing by Means of *galK* Recombineering

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Abstract

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

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

1 Introduction

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

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

- The ability to elicit a strong antiviral and antitumor innate immunity; in essence oHSVs behave as antigen-agnostic anticancer vaccines.
- The ability to synergize with checkpoint blockade, and to confer sensitivity to and widen the activity of checkpoint inhibitors.
- 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 herpesviruses has been critical for development and improvements of technologies aimed at generating HSV recombinants with the desired modifications. Of the various genetic engineering technologies that our laboratory has applied over time, we found that *galK* (galactokinase) recombineering (recombination-mediated genetic engineering) is straightforward, relatively easy to design and to carry out with high rate of success. It requires the prior cloning of the herpesviral DNA as BAC (bacterial artificial chromosome) [8, 9]. The insertion of BAC sequences in HSV was described in detail [10–13] and was covered previously in this book series [14]. *galK* recombineering allows the introduction of the planned genetic modification into HSV-BAC DNA in bacteria (*E. coli*). Of note, even essential viral genes can be readily manipulated, since no viral gene expression takes place in the bacterial host. Following recombineering, recombinant virus can be rescued upon transfection of the genetically modified (recombinant) HSV-BAC DNA in mammalian cells, susceptible and permissive to HSV. Of note, several types of viral modifications can be obtained, including point mutations, deletions/insertions, marker or transgene expression, changes to viral tropism, etc.

The initial technologies for HSV-BAC engineering in *E. coli* (two-step replacement strategy) involved the RecA-mediated homologous recombination of the HSV-BAC with a transgene carried by a shuttle plasmid and the use of two different markers to select for the cointegrate and the resolved final genome [15]. The technology had some pitfalls, mainly because of low stringency of the marker employed in the counterselection of the unresolved recombination intermediates. In particular, the *sacB* marker present in the shuttle vector was quite easily inactivated and lost its efficacy. In addition, the cloning of the desired modified viral gene or heterologous transgene in a shuttle vector prevented

in most cases a seamless insertion of the desired mutations, due to the restriction sites used for cloning.

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

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

In this chapter we provide an example of the insertion of a transgene in HSV-BAC, by means of *galK* recombineering. The subsequent steps for the rescue and cultivation of the recombinant virus are illustrated in a separate chapter of this book (*see* Chapter 8). We highlight the specific problems that can arise in the procedure. The specific example is the generation of R-115, that is, the insertion of mIL12 in R-LM113, an oHSV retargeted to HER2 cancer receptor and expressing the EGFP reporter gene (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 used to harbor and amplify HSV-BACs, and for BAC recombineering using *galK* positive/negative selection [18]. SW102 strain must be grown at a temperature not exceeding 32 °C, to avoid unwanted expression of the three λ Red-encoded genes (*exo*, *bet*, and *gam* recombinases) from a stably integrated defective λ prophage (*see* **Note 1**).

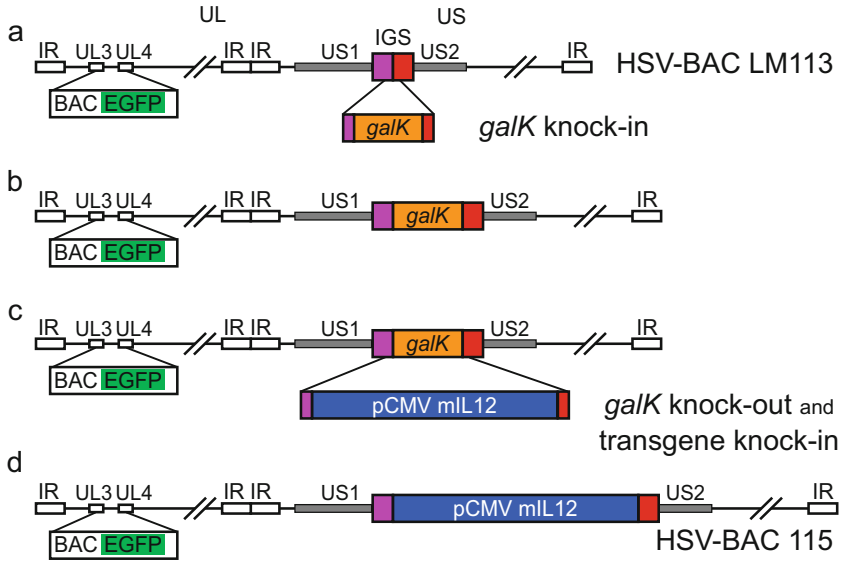

Fig. 1 Schematic diagram of *galK* recombineering for the generation of HSV-BAC 115. The line lengths and box sizes are representative and are not drawn to scale. (**a**) The starting HSV-BAC LM113 carries EGFP as reporter gene (green box). The two halves of the intergenic sequences (IGS) between US1 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

2. *E. coli* containing HSV-BAC DNA (e.g., DH10B containing HSV-BAC LM113 [17]).
3. "Low salt" LB medium (*see* **Note 2**): 10 g Bacto tryptone, 5 g yeast extract, and 5 g NaCl in 1 L ddH_2O. Autoclave for 20 min at 121 °C.
4. Chloramphenicol, 20 mg/mL in EtOH.
5. BAC DNA extraction kit (e.g., NucleoBond® BAC 100 kit (Macherey-Nagel), containing buffers S1, S2, S3, N3, and N5).
6. STE Buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl.
7. Isopropanol.
8. 70% ethanol.

2.2 Buffers and Solutions for galK Recombineering and Characterization of Clones

1. 10× M9 medium: 60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl in 1 L ddH_2O, autoclave.
2. 5× M63 buffer: 10 g $(NH_4)_2SO_4$, 68 g KH_2PO_4, 2.5 mg $FeSO_4{\cdot}7H_2O$ in 1 L ddH_2O, adjust to pH 7.0 with 10 N KOH, autoclave.
3. Supplements for M63 minimal plates: D-biotin (0.2 mg/mL, sterile filtered); D-galactose (20%, autoclaved); 2-deoxy-galactose (DOG, 20%, prepared just before use, sterile filtered, *see* **Note 3**); glycerol (20%, autoclaved); L-leucine (10 mg/mL, dissolved by heating, then cooled down and sterile filtered); $MgSO_4{\cdot}7H_2O$ (1 M, autoclaved); Chloramphenicol (20 mg/mL in EtOH).
4. M63 minimal plates: autoclave 15 g Bacto agar (*see* **Note 4**) in 800 mL of ddH_2O, cool down to 50 °C; add 200 mL of 5× M63 medium and 1 mL of 1 M $MgSO_4{\cdot}7H_2O$; adjust volume to 1 L with sterile ddH_2O if necessary. Add 5 mL biotin (1 mg), 4.5 mL leucine (45 mg), and 625 μL chloramphenicol (12.5 μg/mL working concentration). Complete with the carbon source and/or selective compound: for galactose minimal plates, add 10 mL of 20% D-galactose; for DOG minimal plates, add 10 mL of 20% glycerol and 10 mL of 20% 2-deoxy-galactose.
5. MacConkey indicator plates: prepare MacConkey agar according to the manufacturer's instructions (*see* **Note 5**), autoclave, cool at 50 °C, add D-galactose (1% final concentration) and chloramphenicol (12.5 μg/mL working concentration).
6. High fidelity polymerase and buffer (e.g., Phusion Polymerase (Thermo Fisher)).
7. Taq polymerase and buffer.
8. 50 mM $MgCl_2$.
9. dNTP mix, 10 mM.
10. Restriction endonucleases *Dpn*I, *Bam*HI, *Eco*RV, and *Kpn*I.
11. 1-kb DNA ladder.
12. SK-OV-3, human ovarian cancer cell line (ATCC HTB-77).
13. Lipofectamine 2000.

2.3 Nucleic Acids

1. pgalK for *galK* knockin [18] (Fig. 2a).
2. pLM84, template plasmid for PCR amplification of a pCMV-mIL12 expression cassette for recombination in HSV-BAC [19] (Fig. 2b).

2.4 Agarose Gel Electrophoresis Buffer

1. 50× TAE buffer (1 L): 242 g of Tris base, 57.1 mL of acetic acid (glacial), 100 mL of 0.5 M EDTA (pH 8.0). Working dilution: 0.5×.

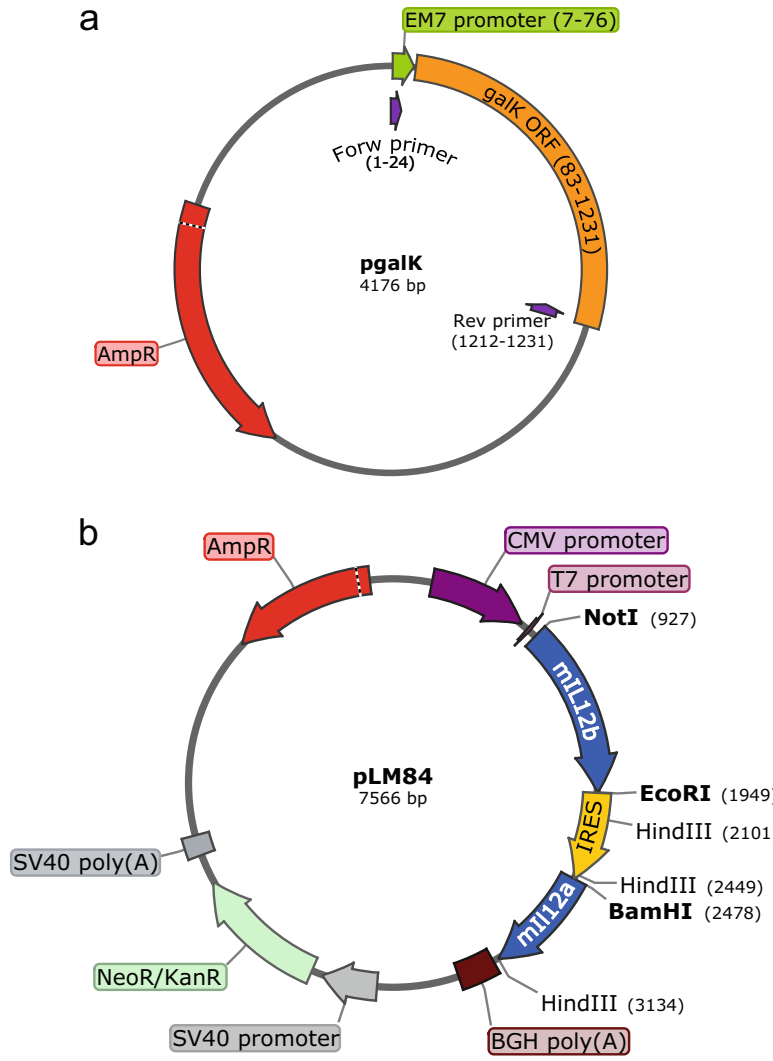

Fig. 2 Maps of the plasmids used for *galK* recombineering. (**a**) pgalK is the template to amplify the *galK* cassette. The positions where chimeric primers anneal are depicted as purple arrows. (**b**) pLM84 is used as template for the amplification of the pCMV-mIL12 cassette [19]. mIL12b (subunit 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.
3. Agarose.

2.5 Equipment

1. Bench instrumentation: benchtop centrifuges, spectrophotometer, electroporation system and electroporation cuvettes (0.2 cm), Speed-Vac.

2. Molecular biology: PCR thermal cyclers, gel electrophoresis system (for short and long run lengths), gel imaging system.
3. Incubators and shakers for bacteria.
4. Folded filter paper.
5. Ice–water slurry.
6. Eppendorf tubes.
7. 50 mL conical tubes.
8. 500 mL bottles.

3 Methods

This collection of protocols describes the methods to modify an HSV-BAC by *galK* recombineering technologies. In the next chapter, we will describe how to rescue the recombinant HSV, and finally evaluate its biological properties in vitro. We focus here on the expression of a heterologous gene, the cytokine murine interleukin 12 (mIL12) in a replication-competent fully retargeted HSV, by insertion of an expression cassette in the non-coding 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 procedure requires SW102 cells, a strain derived from DH10B, engineered with the λ prophage encoding the recombinases, and deleted of the galactokinase gene (*galK*) in the galactose operon [18]. If the HSV-BAC is hosted in a different bacterial strain, its DNA must be extracted and transferred by electroporation into SW102 bacteria.

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

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

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

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) column with 2.5 mL of equilibration buffer (N2). Allow the column to completely empty by gravity flow and discard flow-through.
7. Clarify the lysate through a wet folded filter paper placed in a funnel (*see* **Note 10**); collect the flow-through.
8. Load the cleared lysate onto the equilibrated NucleoBond® BAC 100 column; allow the column to empty by gravity flow, discard flow-through.
9. Wash the column twice with 12 mL wash buffer (N3) and discard flow-through.
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 30 min at 4 °C. Carefully remove and discard the supernatant.
12. Add 1 mL ice-cold 70% ethanol to the pellet of each aliquot, centrifuge at 14,000 × *g* for 10 min at 4 °C. Speed-Vac dry for 10 min.
13. Dissolve each aliquot of HSV-BAC DNA in 10 μL of sterile ddH_2O. Let the pellet hydrate for about 15 min. To avoid shearing of HSV-BAC DNA, pipet gently using wide-orifice tips.
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**).

3.2 Electroporation of HSV-BAC into SW102 Bacterial Strain

1. 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 medium (*see* **Note 15**), without antibiotics, in a bottle or a flask. Measure bacterial density (OD_{600}) at time 0 (it should be at least 0.03). Incubate the culture for about 3–4 h in a shaker at 30 °C (in an incubator or in a water bath) and measure bacterial density at regular time intervals.

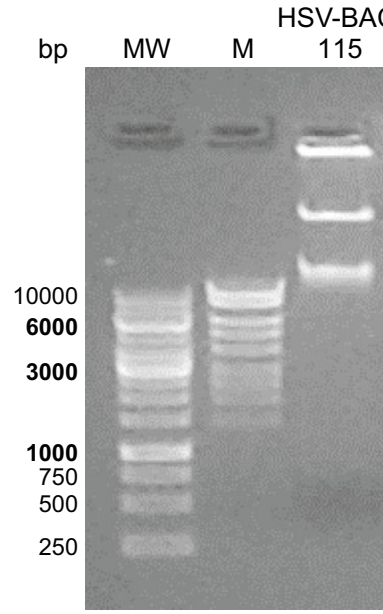

Fig. 3 Typical band pattern of an intact HSV-BAC DNA run on a 0.8% agarose gel in 0.5× TAE. The bands are sharp, without smear. MW: GeneRuler 1 kb DNA Ladder; sizes are in base pairs (bp). M: MassRuler DNA Ladder Mix (Thermo Scientific): *: 50 ng, **: 40 ng

3. During the bacterial growth, prepare an ice–water slurry and cool on ice 1 L of sterile ddH_2O, 50 mL conical tubes, Eppendorf tubes, 0.2 cm electroporation cuvettes (*see* **Note 16**).
4. When the OD_{600} of the culture is near 0.6, the bottle containing the bacteria is cooled down in the ice–water slurry for 1–2 min, and subsequently transferred into precooled 50 mL conical tubes.
5. Spin down the bacteria in a cold (0–1 °C) centrifuge for 8 min at 3000 × *g*.
6. Pour off all the supernatant, then add 5 mL ice-cold ddH_2O, while keeping the tube with the bacterial pellet in the ice–water slurry. Resuspend the pellet in water by gently shaking the tube in the ice–water slurry (the first time it will take about 5 min). When resuspended, fill up to 50 mL with ice-cold ddH_2O, mix by inversion, and spin in a cold centrifuge for 8 min at 3000 × *g*.
7. Make a second cold water wash as in **step 6**, then remove all supernatant by inverting the tube on a paper towel. Gently resuspend the bacterial pellet in the residual small amount of ddH_2O left in the tube and store the competent cells on ice.
8. Transfer 50 μL of the freshly made electrocompetent cells to a pre-cooled Eppendorf tube and mix with 100 ng–1 μg of the

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

9. Transform by electroporation (200 Ω, 25 μF, 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.
10. Plate different amounts (1, 10, or 100 μL, and all the rest) of the transformed bacteria on low salt LB agar plates plus chloramphenicol (12.5 μg/mL) to obtain single colonies (*see* **Note 17**). Incubate at 30 °C for 1–2 days.

3.3 GalK Recombineering

The *galK* recombineering technology allows to modify a BAC DNA cloned in *Escherichia coli* via lambda (λ) Red-mediated homologous recombination. In this strategy, the use of restriction enzymes and DNA ligases to modify DNA is not required, instead BAC DNA is modified using *galK* positive/negative selection in a two-step procedure [18]. The first step is a homologous recombination to insert (knockin) the *galK* cassette into the desired position of the HSV-BAC DNA. Recombinant clones are selected by positive selection of bacteria that acquired the ability to grow on minimal media with galactose as the only carbon source. The second step involves another homologous recombination to substitute the *galK* cassette (*galK* knockout) with the sequence of interest. In this case a negative selection against *galK* with 2-deoxy-galactose (DOG), toxic following phosphorylation by the *galK* gene product, ensures the identification of recombinant clones. Both recombinations occur via short homology sequences or homology arms, which flank the *galK* or the custom cassette, and are homologous to the selected target position in the HSV-BAC DNA (Fig. 1).

3.3.1 GalK Knockin (Positive Selection)

The first step of *galK* recombineering technology consists of the insertion of the constitutively expressed galactokinase cassette (*galK*) into the HSV-BAC locus chosen for the selected modification. To this purpose, first, it is necessary to PCR amplify the *galK* cassette flanked by short homology arms included in the primers (*see* **Note 18**).

1. Design PCR chimeric primers for the amplification of the *galK* cassette. For the insertion described here, we used the following primers: US1/US2_galK_f *ATAAAAGACCAAAATCAAAGCGTTTGTCCCAGCGTCTTAATGGCGGGAAG*CCTGTTGACAATTAATCATCGGCA, and US1/US2_galK_r *AATAAACCCCCAAACACCCCCCATGTACGCGTGGTCTGTTTCTCTCCGCC*TCAGCACTGTCCTGCTCCTT (arms with homology to

HSV-BAC are in *italics*, whereas the sequences annealing to *galK* cassette are in plain text).

2. Set up the PCR reaction with a high fidelity polymerase (here a 50 μL reaction with ThermoFisher Phusion polymerase is reported): 10 μL of 5× Phusion HF buffer, 1 μL of 10 mM dNTPs, 2.5 μL of 10 μM forward and reverse primers (0.5 μM final concentration), 0.5 μL of Phusion polymerase; 33 μL of ddH_2O. Finally, add 1 μL of 2 ng/μL pgalK plasmid as template. Amplification conditions: initial denaturation and hot start at 98 °C for 30 s, then 32 cycles at 98 °C for 10 s, 58 °C for 20 s, 72 °C for 30 s, final extension at 72 °C for 10 min. Run 5 μL of the PCR product on a 0.8% agarose gel. A band of ~1.3 kbp is expected.
3. Digest the PCR product with 2 μL (40 U) of DpnI restriction enzyme/50 μL reaction, for 1 h at 37 °C to remove the methylated template.
4. Run the DpnI digestion on a 0.6% agarose gel (1 h at 80 V) and purify the *galK* band by gel extraction spin columns. Elute the *galK* fragment in 50 μL nuclease free ddH_2O (*see* **Note 19**).
5. Measure the concentration of the *galK* fragment with an UV spectrophotometer and dilute it to a final concentration of 30 ng/μL with ddH_2O.
6. Recombineering for *galK* positive selection. Inoculate a single bacterial colony containing the recipient HSV-BAC (in this example BAC LM113) in 5 mL of low salt LB + 12.5 μg/mL chloramphenicol and incubate with shaking at 30 °C overnight.
7. Dilute 2 mL of the overnight culture in 100 mL of low salt LB + 12.5 μg/mL chloramphenicol and shake it at 30 °C to reach an OD_{600} between 0.55 and 0.65. This step takes about 3.5 h. In the meantime precool in an ice–water slurry 1 L of sterile ddH_2O, 50 mL conical tubes, Eppendorf tubes, 0.2 cm electroporation cuvettes.
8. Divide the culture in two 500 mL bottles. Shake one bottle in a 42 °C water bath for 15 min (*see* **Note 20**) to induce λ prophage recombinases (induced sample), leave the other bottle at 30 °C (uninduced control).
9. Cool the two cultures (hereafter designated as "induced" and "uninduced") on ice for 5 min, transfer them to 50 mL precooled tubes and centrifuge for 8 min at 3500 × *g* at 0 °C.
10. Pour off all the supernatant, add 5 mL of sterile ice-cold ddH_2O and resuspend the pellet, with gentle swirling in an ice–water slurry (*see* **Note 21**). Fill the Falcon tubes up to 50 mL with sterile ice-cold ddH_2O and pellet again.
11. Repeat **step 10**.

12. Invert the tubes on a towel to remove the supernatant completely and gently resuspend the bacterial pellet in the residual small amount of ddH_2O left in the tube by swirling in the ice–water slurry. Keep the competent cells on ice.
13. Mix 50 µL of cells and 1 µL of *galK* fragment (30 ng/µL) in a precooled Eppendorf tube.
14. Transfer and electroporate the DNA-cell mix in a precooled 0.2 cm electroporation cuvette at 25 µF, 2.5 kV and 200 Ω (*see* **Note 22**).
15. Add 1 mL of ice-cold low salt LB medium and recover bacteria for 90 min at 30 °C in a shaker.
16. Wash bacteria (induced and uninduced) with 1× M9 salts (*see* **Note 23**): pellet the culture (~1 mL) for 15 s at 14,000 × *g* and remove supernatant carefully with a micropipette, resuspend pellet with 1 mL of 1× M9 salts.
17. Repeat **step 16**.
18. **Plate different amounts (1, 10 or 100 µL, and all the rest) of** the cultures on M63-D-Galactose minimal plates (*see* **Note 24**).
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.

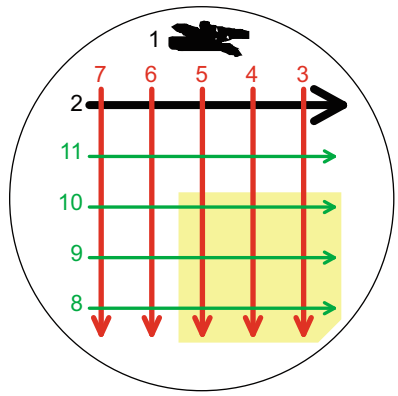

Fig. 4 Dilution of bacteria with the grid method. Pick a single colony with a sterile tip, and deposit the excess on the side of the plate by a forward-and-back streak (black doodle #1). With the same tip make a linear streak as in black arrow #2. Change tip and make linear streaks as depicted by the 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 plate. Repeat the plating with the grid dilution method. This ensures the isolation of pure, non-mixed, clones. Incubate at 30 °C overnight.

22. Choose one or two single brilliant red colonies from each MacConkey plate and make a small linear streak on low salt LB plates + 12.5 μg/mL chloramphenicol. Incubate at 30 °C overnight.

23. Set up a colony PCR reaction to verify the presence of *galK* cassette in the selected position in the HSV-BAC genome. In the example of this chapter we used a forward primer US1_1802_f (ACACGTTTCTCCGGCCGTGAGTCCG) annealing on HSV US1 genomic sequence, and galK_417_r (CATTGCCGCTGATCACCATGTCCACGC) a reverse primer annealing on *galK*, yielding an amplification product of 547 bp. Alternatively, for general purpose *galK* screening, primers both annealing on *galK* sequences can be used (e.g., galK_827_f (GCGTGATGTCACCATTGAAG) and galK_1142_r (TATTGTTCAGCGACAGCTTG)), yielding a 315-bp band (Fig. 5a).

    Taq protocol (20 μL reaction): 2 μL of 10× Taq Buffer, 0.6 μL of 50 mM $MgCl_2$, 0.4 μL of 10 mM dNTPs, 1 μL of 10 μM forward and reverse primers (0.5 μM final concentration), 0.08 μL of Taq polymerase, 14.9 μL of ddH_2O. Finally, as template, pick a tiny amount of bacterial colony, and dissolve it directly in the PCR mix (*see* **Note 27**). When using primers annealing both on *galK*, use 1 μL of 2 ng/μL pgalK plasmid, or a pgalK colony, as positive control. Amplification conditions: initial denaturation at 96 °C for 3 min, then 32 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s, final extension at 72 °C for 10 min.

24. Extract HSV-BAC DNA from four positive clones (*see* Subheading 3.1) and transfect SK-OV-3 (susceptible and permissive) cells with Lipofectamine 2000 (*see* next Chapter, Subheading 3.1) to verify HSV-BAC genome integrity in terms of ability to form plaques, spread and replicate. Monitor the transfected cultures for 3 days for the formation of viral plaques. In our example, plaques are EGFP positive and can be visualized with an inverted fluorescence microscope.

25. Highly recommended: to check the insertion at the intended position, PCR amplify the *galK* cassette including upstream and downstream flanking regions from HSV-BAC genome, and determine DNA sequence.

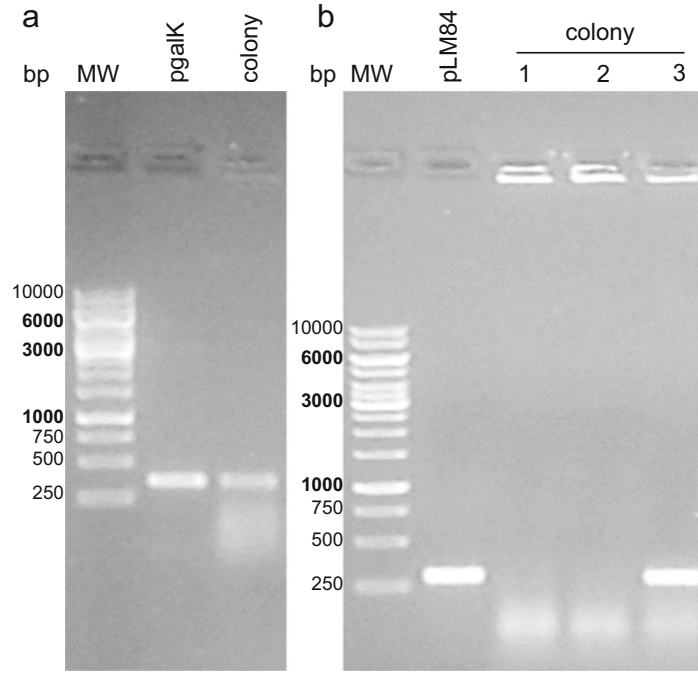

Fig. 5 Colony PCR pattern for *galK* or heterologous mIL12 cassette. Where a plasmid is used as template for the positive control ("pgalK" or "pLM84") the bands are clean and sharp. Where colonies are used as template, below the specific bands a nonspecific halo is visible, due to the dirty input of 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 (Negative Selection)

The second step of *galK* recombineering technology consists in the insertion of the transgene (in this example mIL12) in place of *galK* cassette in the HSV-BAC clone obtained with the first step of recombination. The cassette with the transgene of interest is amplified by PCR including in the primers short arms with homology to the target the insertions site (Fig. 1).

1. Design PCR chimeric primers for the amplification of the transgene cassette. For insertion of mIL12, we used the following primers: US1/US2_CMV_f
   *ATGTCCCCAAATAAAAGACCAAAAT-CAAAGCGTTTGTCCCAGCGTCTTAATGGCGG-GAAG*CGTTTTGCGCTGCTTCGCGATGTACGGGC, and US1/US2_polyA_rev
   *CCCCGATGTCAATAAACCCCCAAACACCCCC-CATGTACGCGTGGTCTGTTTCTCTCCGCC*GCCATA-GAGCCCACCGCATCCCCAGCATGCCTG (arms with homology to HSV-BAC are in *italics*, whereas the sequence

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

2. Set up the PCR reaction with a high fidelity polymerase (here a 50 μL reaction with ThermoFisher Phusion polymerase is reported): 10 μL of 5× Phusion HF buffer, 1 μL of 10 mM dNTPs, 2.5 μL of 10 μM forward and reverse primers (0.5 μM final concentration), 0.5 μL of Phusion polymerase, 33 μL of ddH_2O. Finally, add 1 μL of 2 ng/μL pLM84 plasmid as template. Amplification conditions are: initial denaturation and hot start at 98 °C for 30 s, then 32 cycles at 98 °C for 10 s, 60 °C for 40 s, 72 °C for 4 min. Check the PCR product on a 0.8% agarose gel.
3. Digest the PCR product with 2 μL (40 U) of DpnI restriction enzyme/50 μL reaction, for 1 h at 37 °C to remove the methylated template.
4. Run the DpnI digestion on a 0.6% agarose gel (1 h at 80 V) and purify the band of the heterologous cassette by gel extraction spin columns. Elute the fragment in 50 μL nuclease free ddH_2O (*see* **Note 19**).
5. Quantify the transgene fragment with an UV spectrophotometer and dilute it to a final concentration of 200 ng/μL with ddH_2O.
6. For recombineering and *galK* negative selection, inoculate one *galK* positive clone in 3 mL of low salt LB + 12.5 μg/mL chloramphenicol (in this example, BAC LM113 with *galK* inserted at US1–US2 intergenic region).
7. Prepare electrocompetent cells as in Subheading 3.3.1, **steps 7–12**.
8. Mix 50 μL of cells and 1 μL of transgene fragment (200 ng/μL) in a precooled Eppendorf tube (*see* **Note 28**).
9. Transfer and electroporate the DNA-cell mix in a precooled 0.2 cm electroporation cuvette at 25 μF, 2.5 kV and 200 Ω (*see* **Note 22**).
10. Add immediately 1 mL of ice-cold low salt LB, then transfer to a tube containing 9 mL of low salt LB at room-temperature. Recover bacteria for 4.5 h at 30 °C in a shaker (*see* **Note 29**).
11. Pellet 1 mL of culture and wash twice in 1× M9 salts as in Subheading 3.3.1, **steps 16** and **17**.
12. Plate different amounts (1, 10 or 100 μL, and all the rest) of the induced and uninduced cultures on M63 DOG minimal plates for selection *against galK* (*see* **Note 24**).
13. Incubate at 30 °C for 5–7 days (*see* **Note 30**).
14. Screening of recombinant HSV-BAC DNAs after selection on M63-DOG plates. Choose 30 single colonies from induced

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

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.
16. Choose one or two single white/colorless colonies from each MacConkey plate and make a small linear streak on low salt LB plates + 12.5 μg/mL chloramphenicol. Incubate at 30 °C overnight.
17. Set up two colony PCR reactions to verify the presence of the transgene cassette in the expected position in HSV-BAC genome and the absence of *galK* cassette. In our example we used mIL12a_601_f (CATCCTGCTTCACGCCTTCAGCACCC) and US2_short_r (AACCCCACCCAGCTACCCCAGGCC) for the presence of mIL12 (expected fragment length: 607 bp), and US1_1802_f and galK_417_r (expected fragment length: 547 bp) to verify the absence of *galK*. General purpose primers for the mIL12 heterologous cassette are mIL12b_937_f (CAAAGGCGGGAATGTCTGCGTGC) and IRES_201_r (GGGTTCCGCTGCCTGCAAAGGGTCG) (Fig. 5b).
18. Extract the HSV-BAC DNA from four verified clones (*see* Subheading 3.1), positive for the transgene cassette and negative for *galK*, and transfect them with Lipofectamine 2000 (*see* next Chapter, Subheading 3.1) in the appropriate susceptible and permissive cell line (in this example SK-OV-3). Monitor for the formation of plaques for 3 days.
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. After restriction digestion, for each construct a peculiar restriction enzyme fragments pattern is expected. If possible, include the parental HSV-BAC as control.

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

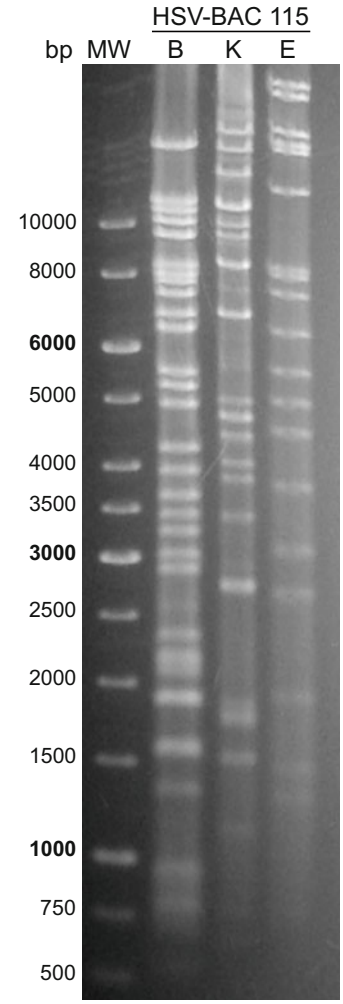

Fig. 6 Check of HSV-BAC 115 DNA integrity by restriction endonuclease analysis with BamHI (B), KpnI (K), and EcoRV (E). Bands were separated on a 0.6% agarose gel in 0.5× TAE. MW: GeneRuler 1 kb DNA Ladder. Sizes are in base pairs (bp)

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

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

3. Prepare just the required amount of 20% 2-deoxy-galactose, to avoid oxidation of the solution. Autoclaving is possible, but not recommended.
4. It is critical to use reliable Agar devoid of any carbon source, in order to perform a stringent selection for *galK*-positive or *galK*-negative clones.
5. Again, it is pivotal to use MacConkey Agar Base totally devoid of lactose or any other carbon source whatsoever, in order to accurately differentiate *galK*-positive and *galK*-negative clones.
6. NucleoBond® PC 100 KIT is suitable for HSV-BAC DNA preparation, as well.
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.
8. The STE wash is recommended to remove any trace of culture medium and improve the purity of the extracted HSV-BAC.
9. Do not vortex, to avoid contamination by bacterial chromosomal DNA released from cellular debris.
10. This step is crucial to avoid clogging of the column in the next step.
11. Preheating highly improves elution and recovery of HSV-BAC DNA.
12. This helps in the subsequent steps of precipitation, washing, drying and resuspension.
13. Avoid freezing at −20 °C: freeze-thaw cycles fragment HSV-BAC DNA.
14. All the procedures of this protocol must be performed with standard aseptic technique close to a Bunsen burner.
15. To increase electroporation efficiency, it is important to reduce NaCl traces in the final suspension of bacterial cells to be electroporated.
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 the transformed culture (the pellet from about 890 μL), too.
18. Typical homology arm length is 50 bp. In case of the insertion of large fragments (3 kb or more) or to increase the recombineering efficiency, it can be extended up to 70 bp for

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

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

26. Pay attention to streak the excess of colony on the tip on a side of the plate, or you will not obtain single colonies.
27. Do not exceed with the quantity of input bacterial colony or the PCR reaction may be inhibited. It is best to spread the bacteria first on the wall of the tube, then to push them into the PCR mix, to avoid lumping and sticking of bacteria on the tip. Alternatively, dilute a colony in 20 μL of ddH_2O, boil for 5 min and use 2 μL as template for the colony PCR reaction.
28. In case of a large transgene cassette, it may be useful to perform electroporation with up to 400 ng of PCR fragment.
29. In the second step of *galK* recombineering the recovery time and the volume are increased with respect to the first step.
30. Small bacterial colonies will appear for both induced and uninduced sample. This happens because the negative selection (*galK* counterselection) step is less stringent than the first positive selection step. For this reason a large number of colonies (sometimes 100+) need to be screened to find recombinant clones.

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