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Protocol of a Seamless Recombination with Specific Selection Cassette in PCR-Based Site-Directed Mutagenesis

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1. Introduction

Genetic mutation has made great contributions to determining protein structure and function, viral pathogenesis, biological engineering, and vaccine development [1-9]. In order to alter genetic information, gene mutation (also called mutagenesis) can be achieved by many different methods. The process of mutagenesis can occur naturally, as the root of evolution; by mutagens, such as chemicals or radiation; and experimentally, by laboratory techniques [10-13]. Current experimental mutagenesis methods can be generally classified into random and directed mutation.

Experimentally directed mutagenesis methods include: insertional mutagenesis [14], PCR mutagenesis [15, 16], signature tagged mutagenesis [17], site-directed mutagenesis [18, 19] and transposon mutagenesis [20-22]. Different procedures are involved in traditional mutagenesis, including molecular cloning that depends on preparations of vector and inserted DNA fragments and ligations. This traditional mutagenesis method is not only time-consuming, but also labor intensive, and modern advancements in mutagenesis have overcome these obstacles. The development of the Bacterial Artificial Chromosome (BAC) clone of large DNA viruses was a breakthrough in the viral mutagenesis field. The BAC clone of a virus can be maintained stably and propagated inside a bacterial cell, which allows for easy manipulation of the viral genome. Any required mutation can be easily and rapidly achieved inside the *E. coli* cell and this mutation can be verified before making recombinant virus [23-27]. Since the development of this novel method for construction of recombinant viruses, detailed functional study of virus genome has been done using this specific BAC recombinatory mutagenesis approach [23-27].

Recombination is an important procedure in experimental mutagenesis. There are two recombination systems that are often used: DNA sequence-specific recombinase-driven recombination and homologous recombination. The DNA sequence-specific recombinase-driven recombination includes Cre-Lox and FLP-FRT recombination systems [28-36]. Cre-

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Lox system depends on Cre recombinase that specifically recognizes lox P (locus of X-over bacteriophage P1) sites, 34-basepair (bp) specific sequences and results in recombination by the excision of intervening DNA sequence and joining of site-specific ends. FLP-FRT involves the recombination of sequences between short flippase recognition target (FRT) sites (also a 34-bp DNA sequence) by the flippase recombination enzyme (FLP or Flp) derived from *Saccharomyces cerevisiae* yeast. After recombination, one copy of the specific DNA sequence will remain in the vector, which will cause potential problems for further mutagenesis, DNA stability and, therefore, is not suitable for some applications, such as vaccine development. In addition, BAC sequence excision methods should be carefully considered, depending upon the design of the BAC. For example, it will become problematic if both the BAC vector and gene-specific antibiotic marker contain the same flanking excision sequences (such as loxP) because this is likely to cause the removal of a much larger sequence of genomic DNA than originally intended.

Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two identical sequences of DNA [37, 38]. Wild-type *E. coli* is ineffective at inducing homologous recombination in foreign DNA because linear DNA is commonly degraded by RecBCD exonuclease. In order to circumvent this problem, SW102 strains were developed that contain a temperature-sensitive λ prophage encoding one gene to temporarily repress RecBCD (*gam*), as well as two genes (*exo* and *beta*) utilized for homologous recombination via double-strand break repair [38]. More specifically, exonuclease degrades DNA from the 5' end of double-strand break sites, while Beta binds to and protects the 3' from further degradation [39, 40]. These overhangs from double-strand breaks allow recombination between viral and plasmid DNA. Because the λ phage is temperature sensitive (due to the expression of a temperature-sensitive λ *ci*-repressor), linear DNA uptake and recombination can occur within a few minutes when the cell-culture temperature is increased from 32 to 42°C [38]. This allows the bacterial cells to function normally when grown at 32°C. *E. coli* also require thousands of homologous base pairs in order for recombination to occur. Addition of the modified temperature-sensitive λ phage is important because this allows homologous recombination to occur within a relatively small region of the homologous sequence, which is important because BAC mutants are usually created using PCR-amplified gene sequences with about 40 base pairs of flanking sequences that are homologous to the viral BAC [37].

PCR-based preparation for insertion of DNA provided convenience in performing mutagenesis studies. Differential PCR techniques enable researchers to make any mutation, as needed: large deletion, point mutation, substitution, insertion, non-sense mutation and shift-mutation. Allowing for the use of a positive and negative selection system is another advancement in the area of mutagenesis, especially when applied to site-directed mutagenesis when accuracy and precision are of high priority. There are multiple ways to carry out site-directed mutagenesis of a viral BAC. For example, selectable markers are necessary to isolate the successful mutation of a viral BAC, and selectable markers can be an antibiotic resistant gene (such as kanamycin or zeocin resistant genes) or a foreign metabolic gene (such as *galK*). In the case of a gene knockout, the foreign DNA usually contains a selectable marker flanked by a DNA sequence homologous to the flanking regions for the gene of interest. Around 40 bp of a flanking

sequence are typically used for homologous recombination. The viral BAC and the marker with homologous sequences are then inserted into *E. coli* via electroporation. Colonies with the BAC recombinant virus can be selected based upon selection markers and verified by PCR [27].

This protocol describes the use of the *galK* positive- and counter- selection schemes to make gene mutations (*e.g.* point mutations, deletions, and insertions) within viral BACs. This system includes only two steps of recombination within a modified bacterial strain, SW102 [41], using selection and counter-selection media, and easily designed PCR. The SW102 strain, derived from DY380 *E. coli* [38, 42, 43], differs from DY380 only in that the galactokinase (*galK*) gene of the galactose operon is defective in SW102 strain. When SW102 bacteria are incubated on minimal media with galactose as the sole source of carbon, the bacteria cannot grow without *galK* supplied *in trans* [41]. When the *galK* gene is provided *in trans*, in this case it replaces a gene of interest, it can complement the defective *galK* gene in the bacteria and the bacteria can grow in minimal media with galactose, therefore, the first step is a positive selection. Later in the protocol, when *galK* is removed from the viral BAC and replaced with a mutated version of the original gene, a counterselection takes place in that clones containing *galK* will be negatively selected for by growing the bacteria on medium with a substance that produces a toxic intermediate only when a functional *galK* is present.

This protocol explains in detail how to use the *galK* positive and counterselection strategies to make any desired mutations (*e.g.* point mutations, deletions, and insertions) based on plasmid or BAC. The modified plasmid or BAC will not contain any extra DNA sequence; therefore, it is referred to as a “seamless” mutation.

2. Materials and methods

Bacterium: *E. coli* SW102 strain (free reagents from Biological Resources Branch of NCI-Frederick) [41]

Plasmid: *pgalK* (free reagents from Biological Resources Branch of NCI-Frederick) [41]

Luria-Broth (LB) medium

Tryptone 10 g

Yeast Extract 5 g

NaCl 10 g

Dissolve components in distilled and deionized water (ddH₂O) and adjust the total volume up to 1 liter.

For LB agar: add agar to a final concentration of 1.5%.

Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, from 121-124°C for 15 minutes.

1X M9 medium (1 liter)

6 g Na₂HPO₄

3 g KH₂PO₄

1 g NH₄Cl

0.5 g NaCl

Dissolve components in ddH₂O and make the total volume up to 1 liter. Autoclave

M63 minimal plates

1L 5X M63

10 g $(\text{NH}_4)_2\text{SO}_4$

68 g KH_2PO_4

2.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

Dissolve components in ddH₂O and make the total volume up to 1 liter. Adjust to pH 7 with KOH.

Autoclave

Other Reagents

0.2 mg/ml D-biotin (sterile filtered) (1:5000)

20% galactose (autoclaved) (1:100)

20% 2-deoxy-galactose (autoclaved) (1:100)

20% glycerol (autoclaved) (1:100)

10 mg/ml L-leucine (1%, heated, then cooled down and sterile filtered)

25 mg/ml chloramphenicol in Ethanol (1:2000)

1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1:1000)

Procedure of making the M63 minimal plates:

1. Autoclave 15 g agar in 800 ml H₂O in a 2-liter flask.
2. Add 200 ml autoclaved 5X M63 medium and 1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
3. Adjust volume to 1 liter with H₂O if necessary.
4. Let cool down to 50°C ("hand touchable hot"), add 10 ml carbon source (final concentration 0.2%), 5 ml biotin (1 mg), 4.5 ml leucine (45 mg), and 500 µl chloramphenicol (final concentration 12.5 mg/ml) or other appropriate antibiotics. Pour the plates, 33-40 plates per liter.

MacConkey indicator plates:

Prepare MacConkey agar plus galactose according to manufacturer's instructions. After autoclaving and cooling to 50°C, to one liter, add 500 µl chloramphenicol (final concentration 12.5 mg/ml) or other appropriate antibiotics, and pour the plates, 33-40 plates per liter.

3. Protocol

3.1 Preparing SW102 that harbors plasmid or BAC

Select the plasmid or BAC that contains the target gene and will be used as a vector for making mutations. Information about the vector, including antibiotic-resistance and the targeted gene, needs to be known. For example, to make any mutation of a gene in murine cytomegalovirus (MCMV), the BAC of SM3fr [46] is usually used. SM3fr contains whole genome of MCMV with chloramphenicol resistance. The DNA sequence and gene structure of SM3fr have been published and can be accessed in public gene bank.

3.1.1 Preparation of electrocompetent cells (Fig. 1. 1a)

1. A 5-ml overnight culture of *E. coli* SW102 in LB medium will be prepared either from the frozen stock or from a single colony at 32°C or lower. The SW102 strain is resistant

- to tetracycline (12.5 µg/ml), it is not necessary, but safe to include tetracycline in this step in order to exclude any possible contamination.
2. The overnight LB culture of SW102 will be diluted by 1:50 by adding 0.5 ml of the overnight culture to an autoclaved 50 ml Erlenmeyer baffled flask with 25 ml LB, but first save 1 ml LB that will be used as a reference for measuring the OD_{600nm}. The diluted SW102 in the flask will be incubated for 3-5 hrs with appropriate antibiotic selection in a 32°C shaking incubator until the density reaches an OD_{600nm} of 0.6. At this point, a bottle of ice-cold 10% glycerol or autoclaved ddH₂O needs to be prepared. (If the competent cells are to be used right away, use autoclaved ddH₂O). The competent cells can be used right away or stored at -70°C for later use.
 3. When the OD_{600nm} is 0.6, the flasks containing the bacteria will be cooled down in the ice water bath slurry for a minute or two and subsequently transferred into pre-cooled 15 ml Falcon tubes.
 4. The bacteria will be centrifuged in a cold centrifuge (4°C) for 5 min at 5000 RPM (standard Beckman or Eppendorf centrifuge).
 5. All supernatant will be poured off and the centrifuge tubes will be briefly inverted on a paper towel, and 1 ml ice-cold ddH₂O or 10% glycerol will be added to resuspend the pellet. And the tube will be kept in the ice. The pellet will be resuspended in the ddH₂O or 10% glycerol by gently shaking the tube in the ice-water bath (gently move the tubes around in circles while keeping them in the ice water slurry, this can take a while for the first time). When the cells are completely resuspended, another 9 ml ice-cold ddH₂O or 10% glycerol will be added, the tube will be inverted for a couple of times, and centrifuged again for 5 minutes.
 6. The supernatant will be poured off and the pellet will be resuspended with 10 ml ice-cold ddH₂O or 10% glycerol, as in Step 5, resuspension should be much faster this time).
 7. The resuspended cells will be centrifuged once more, as in Step 5.
 8. The supernatant will be completely removed by inverting the tube on a paper towel (be careful that you do not lose the pellet). The pellet will be resuspended in 250 µl ice-cold 10% glycerol. The competent cells can be used immediately for transformation or aliquoted and stored at -80°C (volume should be around 50 µl).

3.1.2 Transformation by electroporation (Fig. 1. 1b)

1. Plasmid or BAC DNA will be transformed into the SW102 competent cells made in Step 1.1. The freshly made or stored electrocompetent cells will be mixed with 1-5 µg DNA. The mixture of cells and DNA will be transferred to a pre-cooled 0.1 cm cuvette. It is important that the DNA solution contains low salt because high salt can cause electric shock during electroporation.
2. The cuvette will be placed into the electroporator power source and cuvette holder (Bio-Rad). The conditions for transformation are set according to the strain. For SW102 cells, use 25 mFD, 200 W, and 1.8 kV. The time constant (tau value) should be 3-4 msec. After the electroporation, the bacteria will be transferred immediately to a tube with 1 ml LB medium and incubated at 32°C in a shaking water bath for 1 hr.
3. The incubated bacteria will be smeared onto LB agar plates that contain appropriate antibiotics. The transformed bacteria on selective LB agar will grow as colonies after incubation at 32°C for 18-24 hrs.

4. The generated SW102 harboring plasmid or BAC will be verified by picking up a colony, isolating the DNA by BAC Miniprep (see Step 1.3.) and detecting the DNA with PCR analysis of the BAC (Fig. 3) and/or restriction enzyme digestion (Fig. 4). This plasmid- or BAC-harboring SW102 will be given a name and used to make desired mutation of the target gene. For example, if the SW102 harbors MCMV BAC SM3fr, it will be called SW102_SM3fr.

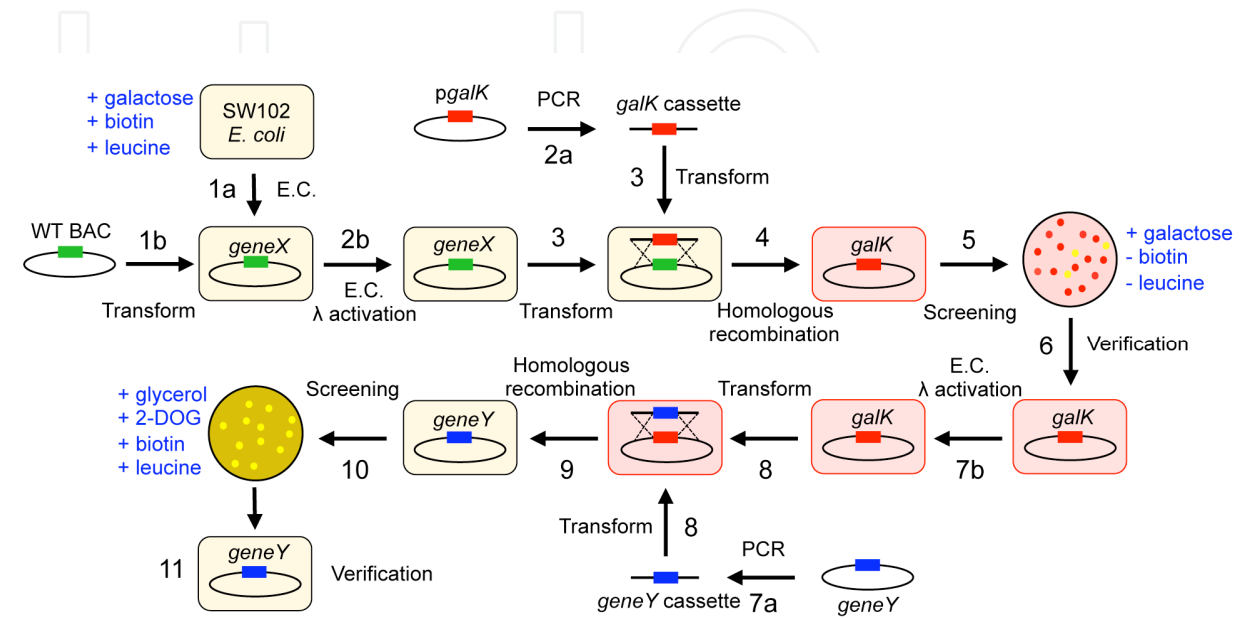


Fig. 1. **Summary of the *galk*-based mutagenesis in *E. coli* SW102.** **1a.** Prepare electrocompetent (E.C.) SW102 *E. coli*. **1b.** Electroporate WT virus BAC into electrocompetent SW102. **2a.** Prepare *galk* cassette by PCR with a set of primers conferring sequence homology to the viral BAC sequences flanking *geneX*. **2b.** Prepare electrocompetent SW102 *E. coli* harboring WT viral BAC and activate defective λ phage recombination system by shaking in a 42°C water bath for 15 minutes. **3.** Electroporate the *galk*-expressing cassette into recombination-activated electrocompetent SW102 strain harboring WT BAC. **4.** Upon homologous recombination, *geneX* is replaced by *galk*. **5.** Confirm presence of *galk* by growing bacteria on M63 plates with galactose as the sole source of carbon and antibiotic, selecting colonies to screen on MacConkey agar with galactose as the sole source of carbon. The *galk*-containing recombinant clones will produce red colonies on MacConkey agar with galactose. **6.** Select red colony from screening process to verify by PCR and continue. **7a.** Prepare *geneY* cassette (PCR cassette containing desired mutation in *geneX*, referred to as *geneY*). **7b.** From red colony selected in Step 5, prepare electrocompetent SW102 harboring *galk* mutant BAC and activate defective λ phage recombination system, as in 2b. **8.** Electroporate *geneY* cassette into electrocompetent and recombination-activated SW102 containing *galk* mutant clone. **9.** Upon homologous recombination, *galk* is replaced by *geneY*. **10.** Grow bacteria on M63 with glycerol, DOG and antibiotic agar plates. **11.** Select colony and verify for production of virus.

3.1.3 BAC miniprep

The following protocol is usually used and works very well for generating BAC DNA for initial analysis:

1. 5 ml overnight LB culture with chloramphenicol (almost all known BACs are chloramphenicol resistant) in a 15-ml Falcon tube is pelleted, and the supernatant is removed.
2. Then the pellet is dissolved in 250 µl buffer P1 (Miniprep kit, Qiagen, CA) and transferred to an Eppendorf tube.
3. The bacteria are lysed in 250 µl P2 buffer with gently mixing and incubating for 5 min at room temperature.
4. The lysate is neutralized with 250 µl buffer P3 (also called N3 buffer), followed by mixing and incubating on ice for 5 min.
5. The supernatant is cleared by two rounds of centrifugation at 13,200 RPM for 5 min in a small Eppendorf centrifuge (or other model tabletop centrifuge). Each time the supernatant is transferred to a new tube.
6. The DNA is precipitated by adding 750 µl isopropanol, mixing and incubating on ice for 10 min, and centrifugation for 10 min at 13,200 RPM in a small Eppendorf centrifuge (or other model tabletop centrifuge).
7. The pellet is washed once in 70% ethanol and the air-dried pellet is dissolved in 50-100 µl TE buffer. The isolated BAC DNA can be used for 1) restriction analysis, 2) PCR analysis and 3) DNA sequencing analysis.

3.2 Positive selection to replace the targeted DNA with *galK* gene

3.2.1 PCR to generate the *galK* cassette (Fig. 1. 2a)

1. Design primers with 50 bp homology flanking the desired site to be modified. The 3' end of these primers will bind to the *galK* cassette. For example, if a single base pair (bp) mutation will be generated, the homology arms should extend 50 bp on either side of the target bp. If the target bp is not included in the arms, it will result in a deletion of that base pair in the first step. If the target bp was changed into another bp in the arm, it will result in single bp mutation. If a small or a large deletion will be made, design the *galK* primers so that the deletion is made already in the first step. The primers should be as follows:

Forward: 5' 50bp homology: CCTGTTGACAATTAATCATCGGCA-3'

Reverse: 5' 50bp homology complementary strand: TCAGCACTGTCCTGCTCCTT-3'

2. Plasmid *pgalK* will be used for PCR to amplify the *galK* gene using the primers designed as above and a proofreading DNA polymerase. It is important to use this type of DNA polymerase because the two-step substitution in the procedure will use PCR products and accuracy is a high priority. DNA template for PCR is the *pgalK* plasmid. PCR can be started at 94°C for 4 min to denature the template, followed with 30 cycles of three temperatures: 94°C, 15 sec; 60°C, 30 sec; 72°C, 1 min; and the PCR will be extended for 7 min at 72°C.
3. When the PCR is finished, 1-2 µl DpnI (New England Biolabs, MA) should be added into each 25 µl reaction that is mixed and incubated at 37°C for 1 hour. This step serves to remove any plasmid template; plasmid is methylated so that DpnI can degrade it, PCR products are not methylated so DpnI cannot degrade it. Finally, the DpnI-digested PCR product will be separated in agarose gel and purified from gel. The PCR product will be eluted with 50 µl ddH₂O, 10-30 ng will be used for transformation.

3.2.2 Preparation of electrocompetent cells harboring BAC

SW102 *E. coli* strain has a defective λ prophage that, when activated, can produce recombinase. These activated and electrocompetent SW102 harboring plasmid or BAC DNA (e.g. SW102_SM3fr) (Fig. 1. 2b) will be prepared from the electrocompetent SW102 harboring plasmid or BAC DNA prepared in Step 1.2 of the protocol. The procedure is as follows:

1. Inoculate an overnight culture of SW102 cells containing the BAC in 5 ml LB with appropriate antibiotics (e.g. SW102_SM3fr in LB with chloramphenicol) at 32°C.
2. Next day, add 0.5 ml of the overnight SW102 harboring plasmid or BAC DNA in 25 ml LB with antibiotics in a 50-ml baffled conical flask and incubate at 32°C in a shaking water bath to an OD_{600nm} of approximately 0.6 (0.55-0.6). This usually takes 3-4 hrs. During this time, turn on two shaking water baths: one at 32°C, the other at 42°C. Make ice/water slurry and pre-chill 50 ml of ddH₂O.
3. When the OD_{600nm} of SW102 harboring plasmid or BAC DNA culture reaches 0.6, transfer 10 ml of the culture to another baffled 50-ml conical flask and heat-shock at 42°C for exactly 15 min in a shaking water bath. This step is to induce SW102 to produce recombinase. The remaining culture is left at 32°C as the un-induced control.
4. After 15 min induction, the SW102 in two flasks (10 ml induced and 10 ml un-induced) are briefly cooled in ice-water bath and then transferred to two 15-ml Falcon tubes and centrifuged at 4°C. It is important to keep the bacteria as close to 0°C as possible in order to get high efficiency competent cells.
5. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold autoclaved ddH₂O by gently swirling the tubes in the ice-water bath slurry (no pipetting). This step may take a while. When the cells are resuspended, add another 9 ml ice-cold autoclaved ddH₂O. Pellet the samples again as in Step 1.4.
6. Resuspend the pellet again with 1 ml of ice-cold autoclaved ddH₂O by gently swirling and then add 9 ml ddH₂O. The bacteria are centrifuged again, as above.
7. After the second washing and centrifugation step, all supernatant must be removed by inverting the tubes on a paper towel, and the pellet is resuspended in approximately 50 μ l of ice-cold ddH₂O and is kept on ice until electroporated with PCR product. If the electrocompetent cells are prepared with 10% glycerol, the aliquots can be made and saved at -80°C for later use.

3.2.3 Electroporation of *galK* cassette

1. 25 μ l of electrocompetent SW102 cells harboring plasmid or BAC DNA and 10-30 ng of PCR amplified *galK* cassette will be added to a 0.1 cm cuvette (BioRad). Electroporation will be carried out at 25 mF, 1.75 kV, and 200 ohms (Fig. 1. 3). After reaction, 1 ml LB will be immediately added to the cells, and the cells will be cultured at 32°C for 1 hour in a shaking water bath.
2. After the recovery incubation, the bacteria are washed twice with M9 buffer as follows: 1 ml of the culture is centrifuged in an Eppendorf tube at 13,200 RPM for 15 sec. Then the supernatant is removed with a pipette. The pellet is resuspended with 1 ml M9 buffer, and pelleted again. This washing step will be performed once more. After the second wash, the supernatant will be removed and the pellet will be resuspended in 1 ml M9 buffer. A serial of dilutions in M9 buffer will be made (100 μ l, 100 μ l of a 1:10 dilution, and 100 μ l 1:100) and plated onto M63 minimal media plates with galactose,

- leucine, biotin, and appropriate antibiotics (Fig. 1. 5). It is important to remove any rich media from the culture prior to selection on minimal media by washing the bacteria in M9 buffer. The uninduced SW102 will be plated as a control.
3. The plates will be incubated for 3 days at 32°C in a cabinet-type incubator. Colonies will be visible at the beginning of third day and be able to be picked up at the end of that day.
 4. Pick up a few colonies and streak the colonies onto MacConkey agar plates with galactose, indicator and appropriate antibiotics to obtain single colonies (Fig. 1. 5). The colonies appearing after the 3 days of incubation should be *galK* positive, but in order to get rid of any *galK* negative contaminants (usually called hitch-hikers), it is important to obtain single, bright red colonies before proceeding to next step (Fig. 1. 6). *galK* negative colonies will be white or colorless and the *galK* positive bacteria will be bright red or pink due to a pH change resulting from fermented galactose after an overnight incubation at 32°C (Fig. 2).
 5. Pick up a few bright red (*galK* positive) colonies and inoculate in 5 ml LB + antibiotics and incubate overnight at 32°C. There is normally no need to further characterize the clones. But the *galK* positive clones can be Miniprepared (Step 1.3) and verified by PCR using primers that flank the site of the *galK* gene (Fig. 3):

Forward: 5' CTGTTGACAATTAATCATCGGCA-3'

Reverse: 5' TCAGCACTGTCCTGCTCCTT-3'

The recombinant *galK* clones should be named for storage. Take MCMV BAC as an example: SW102_SM3fr_X*galK* is the name of the bacteria and SM3fr_X*galK* is the name of the BAC. "X" stands for the site of the *galK* in the BAC.



Fig. 2. Positive selection for *galK* mutant clones and negative selection for WT/mutant/rescue clones. SW102 bacteria with WT, *galK* mutant (*geneX* replaced by *galK* gene), *geneY* mutant (*galK* replaced by mutated *geneX*), or rescue (*geneX* restored) BACs were plated on MacConkey agar with galactose as the sole source of carbon and antibiotic. Recombinant BAC clones containing *galK* appear as red colonies; clones with WT BAC, *geneY* mutant BAC, or rescue BAC appear as white/colorless colonies.

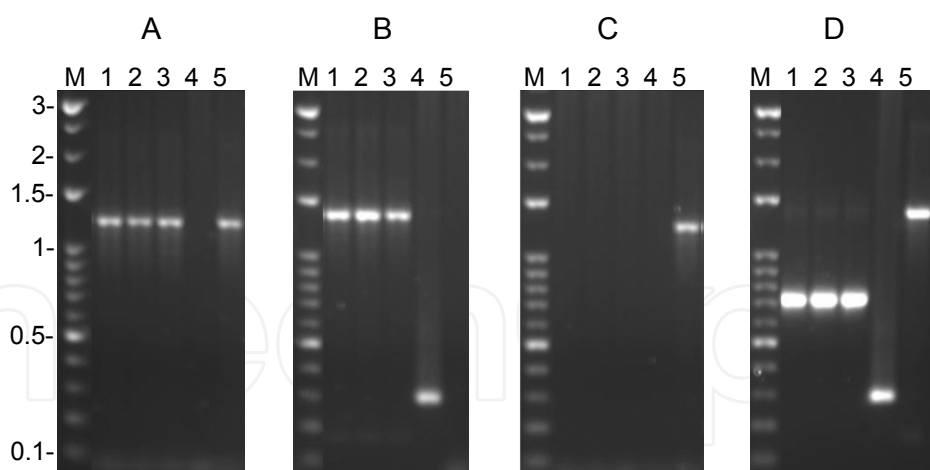


Fig. 3. PCR verification. **A.** PCR using *galK* primers with *galK* mutant clones (*geneX* replaced by *galK* – clones produced from Fig. 1. 4) as template. 1.3kb band indicates presence of *galK* gene: Lanes 1-3 are *galK* mutant clones #1-3, respectively; Lane 4 is original BAC (negative control); Lane 5 is *pgalK* (positive control). **B.** PCR using primers that override the homologous sequences with *galK* mutant clones as template. 1.4kb band indicates presence of *galK*+BAC sequence, 300 bp band indicates BAC sequence only: Lanes 1-3 are *galK* mutant clones #1-3, respectively; Lane 4 is original BAC (positive control); Lane 5 is *pgalK* (negative control). **C.** PCR using *galK* primers with mutant clones (*galK* replaced by *geneY*) as template (mutant clones #1-3 were created, respectively, from *galK* mutant clones #1-3 in Panels 1+2). 1.4kb band indicates presence of *galK*+BAC sequence: Lanes 1-3 are mutant clones #1-3, respectively; Lane 4 is original BAC (negative control); Lane 5 is a *galK* mutant clone used to derive a mutant clone (i.e. *galK* mutant clone #1 from Panels 1+2) (positive control). **D.** PCR using primers that override the homologous sequences with mutant clones as template. 700 bp band indicates presence of mutant gene (*geneY*), 300 bp band indicates WT BAC sequence, 1.4kb band indicates presence of *galK*+BAC sequence: Lanes 1-3 are mutant clones #1-3, respectively; Lane 4 is original BAC (positive control); Lane 5 is a *galK* mutant clone used to derive a mutant clone (positive control). All gel electrophoreses were on 1% agarose gels. M = 1 kb-Opti DNA Marker (ABM, Canada). Marker units are kilobases.

3.3 Counterselection to replace the *galK* gene for desired mutant generation

3.3.1 PCR to generate the DNA fragment to substitute *galK* gene (Fig. 1. 7a)

Design primers with 50 bp homology flanking the desired site to be modified. This 50 bp homology is usually the same as that in making *galK* gene. Usually the mutations are contained in the templates. The DNA template is a WT BAC or plasmid with the gene of interest mutated in the required fashion. The PCR product should therefore contain the desired mutations.

Forward: 5' 50bp homology target gene (18-20 bp)

Reverse: 5' 50bp homology complementary strand target gene (18-20 bp)

3.3.2 Electroporation of DNA fragment (same conditions as in Step 2.2)

1. Both the preparation of electrocompetent cells from the SW102 harboring plasmid or BAC with *galK* gene and the activation of defective λ prophage recombinase are

necessary (Fig. 1. 7b), and the procedure is a repetition of the preparation and activation of electrocompetent SW102 harboring WT BAC or plasmid in Step 2.2.

2. Transform the PCR product into electrocompetent SW102 harboring *galK* mutant plasmid or BAC by electroporation with 0.2-1 µg of PCR product that contains the desired mutation and with homology to the area flanking the *galK* gene (Fig. 1. 8). After electroporation, the bacteria will be recovered in 10 ml LB in a 50 ml baffled conical flask by incubating at 32°C in a shaking water bath for 4.5 hrs. This long recovery period serves to obtain bacteria that only contain the desired recombined BAC or plasmid, and thus have lost any BAC still containing the *galK* cassette.
3. 1 ml of the recovery culture will be centrifuged and the pellet will be resuspended with M9 buffer and washed twice with M9 buffer. After second washing, the pellet will be resuspended in 1 ml of M9 buffer and the bacteria will be plated in a serial dilution (100 µl, 100 µl of a 1:10 dilution and 100 µl 1:100) onto M63 minimal media plates that contain glycerol, leucine, biotin, 2-deoxygalactose (DOG), and appropriate antibiotics (Fig. 1. 10).
4. Incubate at 32°C for three days. Further verification can be accomplished by PCR and/or DNA sequencing (Fig. 1. 11). A name is required for any mutation. For MCMV BAC, we name the bacterial strain SW102_SM3fr_XY and the BAC SM3fr_XY. "X" stands for the site of the mutation in the BAC genome, and "Y" stands for the mutations (deletion, insertion, point mutation or other). For BAC mutagenesis, especially for making any mutation on viruses, a rescue BAC for each mutation is very important.

3.4 Generation of rescue clone of each mutation

The mutagenesis protocol, for some purposes, does not need to progress to this step. However, for some studies, especially for viral mutagenesis, it is necessary to make rescue viruses so that the observed phenotype can be compared and confirmed. The same *galK* method will be used to make a rescue clone that was used to generate the original mutation, except for starting with the mutant BAC and working backwards.

1. The SW102_XY will be used to make electrocompetent cells, using the same procedure as in Step 2.2.
2. The competent cells will be electroporated with the PCR product of *galK* cassette (exactly the same cassette as the one produced in Step 2.1), which will result in SW102_X*galK*.
3. After growth and verification, SW102_X*galK* will undergo electrocompetent cell preparation, as before, and electroporated with a DNA fragment that will be made using the same primers, but with a template that contains no mutation.
4. Therefore the rescue clone is made using the backwards steps. We can also give a name such as SW102_XYRes for the bacteria.

3.5 Maxipreparation of BAC DNA (all recombinant clones)

Now, all of the plasmids or BAC DNA can be isolated for any purpose, *e.g.* in the case of herpesviruses, the BAC DNA needs to be isolated and purified for making viruses. Since BAC is usually a large DNA vector, its isolation and identification are different from that of regular plasmid protocol. Fortunately, several high quality kits have been commercially available for preparation of BAC DNA on a large-scale. In this case, the BAC DNA needs to be extracted from the SW102_XY (or SW102_X as a control) for transfection and production of virus.

1. Select a colony to inoculate 5 ml LB with chloramphenicol (final concentration 12.5 mg/ml) and shake overnight in an incubator at 32°C.

2. The following day, save 200-500 μl of culture to make a stock (see below), and add the remainder of the 5 ml culture to 500 ml LB with chloramphenicol (final concentration 12.5 mg/ml) and shake overnight in an incubator at 32°C. To make a bacterial stock, add 100% glycerol to the saved culture to a final concentration of 15% glycerol and store at -80°C.
3. Use the Nucleobond Maxiprep BAC DNA isolation kit (Clontech Laboratories Inc., CA) to extract BAC DNA from the culture. Because of their large size, BAC DNAs need to be handled in a way that avoids any harsh physical shearing force, including vortexing or passing quickly through fine pipette tips. Freeze-and-thaw should also be avoided.
4. The final DNA products are resuspended in 250 μl sterile ddH₂O and quantified by spectroscopy.
5. BAC DNA solutions should always be stored at 4°C.

3.6 Verification of BAC DNA integrity [Varicella zoster virus (VZV) is used as an example in the following sections]

1. Digest 3 μg of mutant BAC DNA, with WT digestion in parallel as control, with 20 U of restriction enzyme. Example: 3 μl of 1 $\mu\text{g}/\mu\text{l}$ VZV WT BAC DNA, 20 U (1 μl) HindIII restriction enzyme (New England Biolabs, MA), 1 μl 10X NEBuffer 2 (New England Biolabs, MA), 5 μl ddH₂O.
2. Incubate overnight at 37°C and run gel electrophoresis on 0.5% agarose gel. Fig. 4 highlights the pattern observed when digesting WT VZV BAC, ORF7 Deletion VZV BAC, and ORF7 Rescue VZV BAC, respectively.

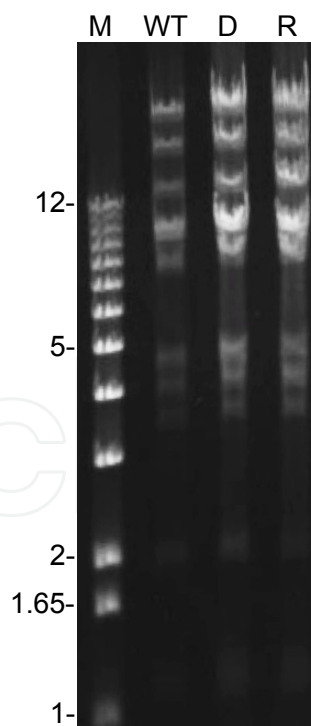


Fig. 4. **Verification of BAC DNA integrity.** For all BACs, 3 μg BAC DNA was digested with 20 U HindIII restriction enzyme overnight at 37°C and ran electrophoretically on 0.5% agarose gel. M: 1 kb Plus DNA Ladder (Invitrogen, CA), units are kb; WT: VZV WT BAC DNA; D: VZV ORF7 Deletion BAC DNA; R: VZV ORF7 Rescued BAC DNA.

3.7 Transfection of BAC DNA for virus production

BAC DNA from Maxi-preparations is transfected into human cells (*e.g.* MeWo, ARPE-19) using the FuGene 6 transfection kit (Roche, Indianapolis, IN), according to manufacturer's standard protocol. 1.5 μg of BAC DNA and 6 μl of transfection reagent are used for a single reaction in one well of 6-well tissue culture plates. Highly concentrated ($>250 \mu\text{g}/\mu\text{l}$) BAC DNA solutions are viscous, and BAC DNA molecules easily precipitate out of the solution when added to transfection reagent solutions. When such precipitation becomes visible, it is irreversible; predictably, the results of the transfection assays are often poor. Therefore, pre-dilute each BAC DNA before gently mixing it with the transfection reagent.

1. For each reaction, 1.5 μg of BAC DNA is diluted in serum-free medium, and the volume of DNA solution is adjusted to 50 μl with the serum-free medium.
2. For each reaction, 6 μl of transfection reagent is combined with 94 μl of medium.
3. Using pipettor tips, gently stir the DNA solution into the transfection reagent.
4. Incubate mixture for 25 minutes at room temperature.
5. Add DNA-transfection reagent solution to culture plate.

3.8 Infected cell culture

1. Transfected cells are grown in a 6-well tissue culture plate in 2 ml DMEM supplemented with 10% fetal calf serum, 100U of penicillin-streptomycin/ml, and 2.5 μg of amphotericin B/ml [47, 48].
2. Upon visualization of infection, Usually viral plaques will be developed and visualized under microscopy as shown in Fig. 5.

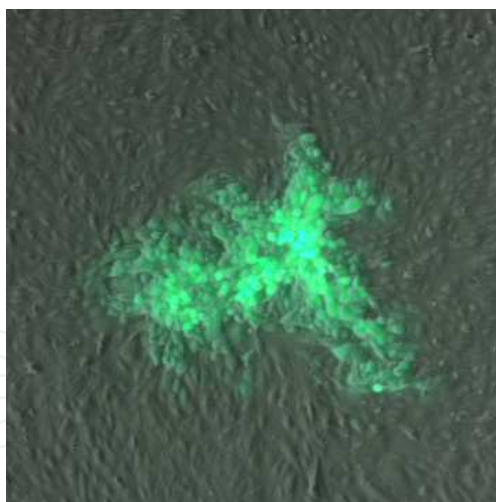


Fig. 5. **Generation of VZV by transfection of viral BAC DNA.** Human ARPE-19 cells were chemically transfected with WT VZV BAC DNA. Infected cells expressing EGFP (inserted into BAC vector) and form green plaques. One plaque is visualized by fluorescent microscope.

3.9 Summary of the protocol

The protocol of the seamless recombination with specific selection cassette in PCR-based site-directed mutagenesis is summarized in Fig. 1, using BAC as an example. Firstly, SW102 *E. coli* is made to be electrocompetent (E.C.) (Fig. 1. 1a). Then, the WT BAC is electroporated

into the electrocompetent SW102 (Fig. 1. 1b). The *galK* cassette is prepared by PCR with a set of primers conferring sequence homology to the viral BAC sequences flanking *geneX* (Fig. 1. 2a). Electrocompetent SW102 *E. coli* harboring WT viral BAC is prepared, and the defective λ phage recombination system is activated by shaking in a 42°C water bath for 15 minutes (Fig. 1. 2b). The *galK*-expressing cassette is electroporated into recombination-activated SW102 strain harboring WT BAC (Fig. 1. 3). Upon homologous recombination, *geneX* is replaced by *galK* (Fig. 1. 4). The presence of *galK* in the recombinant clones is selected by growing bacteria on M63 plates with galactose as the sole source of carbon and the proper antibiotic. Colonies are then selected to screen on MacConkey agar with galactose as the sole source of carbon. The *galK*-containing recombinant clones will produce red colonies on MacConkey agar with galactose (Fig. 1. 5). Fig. 2 highlights the easily perceived selection model of the *galK* mutagenesis approach, as red colonies are indicative of *galK* presence, whereas the colorless colonies do not express *galK*. A red colony from the screening process is chosen to verify by PCR and continue on with the rest of the protocol (Fig. 1. 6). A *geneY* cassette (PCR cassette containing desired mutation in *geneX*, referred to as *geneY*, or other gene) is prepared by PCR with primers conferring homologous sequences to the *galK* region in the mutant BAC (Fig. 1. 7a). From the red colony, SW102 harboring *galK* mutant BAC, selected in Step 5, are prepared to be electrocompetent and recombination-activated as in Fig. 1. 2b (Fig. 1. 7b). The *geneY* cassette is electroporated into the electrocompetent and recombination-activated SW102 strain harboring *galK* mutant clone (Fig. 1. 8). Upon homologous recombination, *galK* is replaced by *geneY* (Fig. 1. 9). Bacteria are grown on M63 with glycerol, DOG and antibiotic agar plates to counterselect for recombinant mutant BACs (Fig. 1. 10). Since the recombinants will now lack *galK*, selection takes place against the *galK* cassette by resistance to 2-deoxy-galactose (DOG) on minimal plates with glycerol as the carbon source (Fig. 1. 10) [27]. DOG is harmless, unless phosphorylated by functional *galK*. Phosphorylation by *galK* turns DOG into 2-deoxy-galactose-1-phosphate, a toxic intermediate [44]. From the resulting DOG-resistant colonies, some will be background colonies, where the bacteria have lost the *galK* cassette by a deletion, and the rest will be truly recombinant clones. Therefore, recombinant colonies containing the modified gene sequence can be quickly selected due to the negative or counterselection of colonies with *galK*, which makes this timesaving system also highly efficient. The resulting recombinant clones will be verified by PCF (Fig. 3) and restriction enzyme digestion (Fig. 4). Transfection of the mutated viral BAC into mammalian cells then produces an infectious virus (Fig. 5) [43].

4. Discussion

Molecular cloning vectors were once plasmids that could only carry and replicate small-sized DNA and have been developed to yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC) and bacteriophage P1-derived artificial chromosome (PAC) that can carry and replicate large DNA molecules. Based on these vectors, recombineering techniques have contributed profoundly to investigating protein structure, function, and to elucidate viral pathogenesis, among others. However, the methods for BAC mutagenesis we use today were not easily attained as the large size of BACs posed a serious obstacle for their exact manipulation, as is required for viral research. To overcome these obstacles, techniques were developed that utilized the power of homologous recombination in order to create recombinant viruses, with the ability to safeguard every step in the process. We can now replace, and therefore delete, large DNA fragments with selectable marker cassettes,

and within the described *galK* mutagenesis system there is also a counterselection model as well, using new systems that circumvent the problems associated with conventional genetic engineering as there is no longer a size restriction as seen when using restriction enzymes or other previous methods [37,44].

As previously mentioned, the generation of both the mutant and rescued BACs within the *galK* system take advantage of counterselection. For all such counterselection schemes, any event that leads to the loss of the counterselectable marker during negative selection will mean the survival of unwanted bacteria, leading to trace amounts of background. In the *galK* system, BAC replication appears to be the epicenter of background formation [41]. Despite the reliable stability of BACs, rare deletions do occur during counterselection, leading to background. Although there is spontaneous deletion background, it is insignificant relative to the great percentage of correct recombinants due to the high frequency of recombination from the λ prophage system. Furthermore, increasing the length of homology arms used for recombination can increase specific homologous recombination efficient and reduce the number of background deletions relative to the increased number of correct recombinants.

So far, most mutagenesis studies using the BAC system require generation of a rescue clone of the mutation to assure that no mutations occur anywhere other than the target site. The procedure of making rescue BAC DNA requires an original DNA fragment to be inserted back into the viral genome. This can be achieved by PCR amplification of the DNA fragment along with homology arms flanking the mutated region and insertion of the amplified fragment back into the mutant genome by homologous recombination. This method is analogous to procedure for generating recombinant mutants, as the only difference lies in the fact that there is no designed point mutation or deletion achieved via PCR. Antibiotic-resistance selection systems are the most extensively used method, however, removal of the antibiotic-resistance gene is not only necessary for functional analysis of a viral gene, but also required for preparing viral vaccine strains. The *galK* mutagenesis protocol outlined above is a seamless mutagenesis model because there is no requirement for further excision of the flanking homologous sequences that contain *LoxP* or *FRT* that might cause the loss of viral DNA during the process of making viruses in mammalian cells, as there is in antibiotic-resistance based mutagenesis. Thus, the process of making mutants by the above-mentioned protocol is considered to be accurate as there are no unintended mutations that could occur as a result, and even if there are, the strong counterselection strategy and troubleshooting strategies help to clear any background mutants.

5. Conclusion

These and other various advantages are responsible for the increased efficacy of the homologous recombination-based method for the construction of recombinant viruses. Outdated procedures for traditional mutagenesis, including molecular cloning that depends on preparations of vector and inserted DNA fragments and ligations, are laboring and time-intensive. The development of the Bacterial Artificial Chromosome (BAC) clone of large DNA viruses was an innovation that advanced the viral mutagenesis field to a new peak for global mutation and pathogenesis studies as any required mutation can be easily and rapidly achieved by the novel recombineering method for construction of recombinant viruses by homologous recombination. This mutagenesis method has led to a great

expansion in the field of molecular research, whereas *galK* mutagenesis takes this recombineering strategy to a heightened level of accuracy and, therefore, results.

6. Future directions

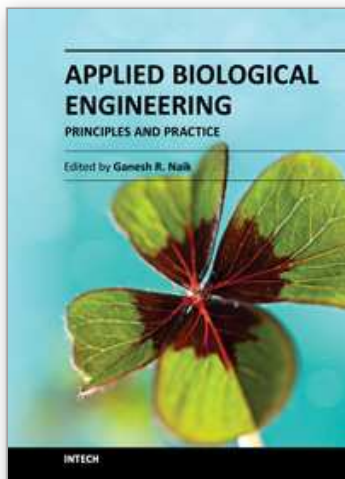
The amount of biomedical research utilizing plasmids and BACs has grown rapidly during the past decade, resulting in invaluable knowledge about protein structure and function, viral pathogenesis, vaccine development and gene therapy. Since the construction of the first herpesvirus BAC 12 years ago, BACs have been generated for all major human and animal herpesviruses, and this technology has greatly facilitated genetic and functional studies of herpesviruses, because recombinant viruses, especially herpesviruses, were previously difficult to produce due to their large size. Soon, we may have BACs for not only all herpesviruses [27], but all DNA viruses, as well as novel global mutational studies for several virus BACs thanks to accurate and seamless mutagenesis procedures such as the *galK* recombineering protocol. Global and local studies of virus pathogenesis should help identify new antiviral targets and produce more effective and safe vaccines. In short, future virus BAC-based mutagenesis studies achieved by the seamless *galK* mutagenesis protocol should help provide exciting new discoveries about viral pathogenesis, protein structure and function, as well as therapeutics for both viral and non-viral diseases.

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Biological engineering is a field of engineering in which the emphasis is on life and life-sustaining systems. Biological engineering is an emerging discipline that encompasses engineering theory and practice connected to and derived from the science of biology. The most important trend in biological engineering is the dynamic range of scales at which biotechnology is now able to integrate with biological processes. An explosion in micro/nanoscale technology is allowing the manufacture of nanoparticles for drug delivery into cells, miniaturized implantable microsensors for medical diagnostics, and micro-engineered robots for on-board tissue repairs. This book aims to provide an updated overview of the recent developments in biological engineering from diverse aspects and various applications in clinical and experimental research.

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