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Using Recombineering to Generate Point Mutations: *galK*-Based Positive-Negative Selection Method

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Abstract

Recombineering is a recombination-based highly efficient method of genetic engineering. It can be used to manipulate the bacterial chromosomal DNA as well as any episomal DNA.

Recombineering can be used to insert selectable or non-selectable DNA fragments, subclone DNA fragments without the use of restriction enzymes and also to make precise alterations including single nucleotide changes in the DNA. Here we describe a *galK*-based two-step method to generate point mutations in the bacterial artificial chromosome (BAC) insert using the recombineering technology. It takes advantage of the ability to select and also counter-select for the presence of *galK*.

Keywords

Recombineering; bacteriophage lambda recombination genes; *galK*; point mutation; bacterial artificial chromosome (BAC); oligonucleotides

INTRODUCTION

Recombineering, genetic engineering using recombination proteins is a powerful system for engineering bacterial chromosomes and episomes *in vivo* by homologous recombination using PCR products and synthetic oligonucleotides as substrates [1]. Francis Stewart and colleagues in 1998 made an important advance in the field of genetic engineering by describing the use of recombination systems encoded by the *recE* and *recT* genes of the λ Red prophage and an analogous λ Red system to manipulate genes [2]. Since then, several different systems have been developed utilizing different recombination machinery [1–3]. This highly efficient technique can be exploited in various ways to manipulate the genome, including the construction of chromosomal gene knockouts, point mutations, deletions, small insertions, *in vivo* cloning, mutagenesis of bacterial artificial chromosomes and genomic libraries [4–8].

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Alternatively the colonies can be first screened by PCR using the primers outside the homology arms. After identifying the PCR positive clones, streak the colonies on the MacConkey agar plates to obtain single *Gal*⁺ colonies. It is important to remove any non-recombinant *galK*⁻ cells before proceeding to step 2 of targeting.

Bacterial artificial chromosome (BAC) is an ideal vector for cloning and manipulating large fragments of DNA [9]. BACs are maintained in *Escherichia coli* (*E. coli*) cells that are *recA*⁻ to ensure the stability of the insert. However, this hinders the manipulation of BACs by homologous recombination in the bacterial cells. To perform BAC recombineering, a bacterial strain that expresses a bacteriophage recombination system is required. The recombination functions are provided by three genes of bacteriophage λ Red locus: *exo*, *bet* and *gam*. *Exo* is a 5′–3′ endonuclease that degrades 5′ ends of linear DNA. *Bet* binds to the single-stranded DNA and promotes annealing to the complementary DNA whereas *Gam* inhibits the *recBCD* exonuclease and protects the DNA from degradation. The defective λ prophage system encoding the *exo*, *bet* and *gam* genes is commonly used for providing recombination functions. The phage recombination systems in the defective λ prophage are under control of bacteriophage λ temperature-sensitive *cI857* repressor. At low temperatures (30° to 34°C), the recombination genes are not expressed. By shifting the bacterial cultures to 42°C, the recombination genes are expressed at high levels from the λ *p_L* promoter.

Here we describe the generation of point mutations, deletions or insertions in BAC DNA using *galK* based positive-negative selection method developed by Warming et al. (2005). This two-step selection allows the modification of BAC DNA without introducing a selectable marker at the modification site (fig. 1). This system uses a bacterial strain containing the λ prophage recombineering system and defective in the utilization of galactose as carbon source due to the deletion of galactokinase gene (*galK*) from galactose operon. The rest of the genes of the galactose operon are intact. So, when the *galK* function is added *in trans*, the ability of *galK*⁻ *E. coli* to grow in a media containing galactose as the carbon source is restored. The *galK* selection scheme is a two-step system: the first step, a positive selection step, involves targeting the region of interest with the *galK* cassette containing homology to a specified position in a BAC. The recombinant bacteria are selected on the minimal plate containing galactose as the carbon source. In the second step, a DNA fragment containing the particular mutation of interest replaces the *galK* cassette. Cells are selected for the loss of *galK* in the presence of 2-deoxy-galactose (DOG) on minimal plates with glycerol as the carbon source. DOG is harmless, unless phosphorylated by functional *galK*. Phosphorylation of DOG by galactokinase turns DOG into 2-deoxy-galactose-1-phosphate, a non-metabolizable intermediate that is toxic to the cells. This is negative selection as it selects the bacteria for the absence of *galK* cassette. This positive-negative selection for BAC manipulation is a highly efficient (60–80%) even with 50 bases of homology on both sides.

2. MATERIALS

2.1 Bacteria and plasmid

***E. coli* SW102:** A modified *E. coli* Dy380 strain [11] containing the defective λ prophage and a fully functional *gal* operon, except for a deletion of *galK*. Loss of *galK* allows for efficient BAC modification using *galK* positive-negative selection. This strain is tetracycline resistant (12.5 ug/ml). This strain is temperature sensitive and must be grown at 32°C.

pGalK plasmid: this plasmid is used as a template to amplify the *galK* cassette by PCR. SW102 and pGalK can be obtained from the NCI-Frederick recombineering resource (<http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>)

2.2 Reagents

Ampicillin (Sigma)

Chloramphenicol (Sigma)

Sterile distilled H₂O, chilled on ice

Plasmid DNA isolation reagents (Qiagen maxiprep and miniprep kits)

PCR purification kit (Qiagen)

Gel extraction kit to purify DNA from agarose gels (Qiagen)

Expand High fidelity Taq polymerase (Roche)

Restriction enzymes (New England Biolabs)

Standard Taq polymerase (Invitrogen)

dNTP mixture, 10mM each, PCR grade, (Invitrogen)

Agarose (SeaKem LE, ISC Bioexpress)

Primers for PCR amplification of recombineering substrates, 25pmol/μl in H₂O (Invitrogen)

2.3 Equipments

Constant temperature bacterial incubator set at 30–34°C (2005 Low temp Incubator, VWR)

Two shaking H₂O baths (200 rpm) set at 30–32°C and at 42°C (New Brunswick Scientific)

Spectrophotometer and cuvettes (Beckman-Coulter)

Electroporator (Genepulser II with Pulse Controller II, Bio-Rad)

Electroporation cuvettes with 0.1 cm gap (BioRad), labeled and prechilled

Floor model low speed centrifuge at 4°C (Avanti, J-25I, Beckman)

Refrigerated microcentrifuge at 4°C (Micromax, RF, IEC)

Thermal cycler and accessories for PCR (MyCycler, BioRad)

Agarose gel electrophoresis apparatus (Bio-Rad)

Sterile 125 and 250 ml Erlenmeyer flasks, preferably baffled (Bellco)

Sterile 35–50 ml centrifuge tubes (Thermo Scientific)

1.5 ml microfuge tubes (Eppendorf)

0.2 ml flat cap PCR tubes (Bio-Rad)

Insulated ice buckets (VWR)

Sterile glass culture tubes (16×150mm) for overnight growth of bacterial cultures (VWR)

Stainless steel closures for culture tubes (VWR)

Pipettors of various volumes (Gilson) with aerosol-resistant sterile tips

Petri plates, 100×15mm (VWR)

2.4 Media

All media were sterilized by autoclave after preparation and stored at room temperature.

M9 salt solution: For 1 liter solution 3.0 g KH_2PO_4 , 12.8 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NH_4Cl , 0.5 g NaCl.

LB (Luria Broth): For 1 liter broth 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl. pH 7.2

“Superbroth special”: For 1 liter broth 35 g Bacto-tryptone, 20 g yeast extract, 5 g NaCl.

SOC medium: For 1 liter media 20 g Bacto-tryptone, 5 g yeast extract, 2ml of 5M NaCl, 2.5ml of 1M KCl, 10ml of 1M MgCl_2 , 10ml of 1M MgSO_4 , 20ml of 1M glucose

M63 minimal plates: For 1L 5X M63 10 g $(\text{NH}_4)_2\text{SO}_4$, 68 g KH_2PO_4 , 2.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Adjust to pH 7 with KOH

Other: 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 EtOH (1:2000)

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

Autoclave 15 g agar in 800 ml H_2O in a 2 liter flask. Let cool down a little. Add 200 ml autoclaved 5X M63 medium and 1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Adjust volume to 1 liter with H_2O if necessary. Let cool down to 50°C (“touchable hot”). Add 10 ml carbon source (final

conc. 0.2%), 5 ml biotin (1 mg), 4.5 ml leucine (45 mg), and 500 ml Chloramphenicol (final conc. 12.5 mg/ml). Pour the plates, 25–40 plates per liter.

MacConkey indicator plates—Prepare MacConkey agar plus galactose according to manufacture s instructions (Difco). After autoclaving and cooling to 50°C, to one liter add 500 µl Chloramphenicol (final conc. 12.5 µg/ml), and pour the plates, 25–40 plates per liter.

3. METHODS

3.1 Preparation of BAC DNA

Identify a BAC containing the gene of interest using a genome browser (like <http://genome.UCSC.edu/>), and order the BAC from Invitrogen or Childre s Hospital Oakland Research Institute's BACPAC Resource (<http://bacpac.chori.org/>). Before proceeding further make sure the BAC contains the gene of interest by PCR analysis and check the integrity of the BAC insert by comparing the restriction digestion pattern (using restriction enzymes such as *EcoRI*, *BamHI*, *PstI* or *SpeI*) of 2 or 3 overlapping BACs.

3.1.1 BAC miniprep—1. Inoculate a single colony of *E.coli* containing the BAC of interest into 10 ml of “superboth special” medium with 12.5 ug/ml of chloramphenicol and grow overnight at 32°C.

2. Pellet down the cells at 6000 g for 5 min. and remove the supernatant.

3. Dissolve the pellet in 200 µl buffer P1 (Qiagen miniprep kit) and transfer to an eppendorf tube.

4. Add 200 µl buffer P2 (Qiagen miniprep kit) and mix the tubes by inversion. Incubate at room temperature for 5 min.

5. Add 200 µl buffer P3 (Qiagen miniprep kit) followed by mixing and incubation on ice for 5 min.

6. Clear the supernatant by centrifuging two times at 12,000 g for 10 min. in a tabletop centrifuge. Transfer supernatant to a new tube each time.

7. Precipitate DNA by adding 600 µl isopropanol to the supernatant and incubating on ice for 10 min. followed by centrifugation at 12,000 g for 10 min.

8. Wash the pellet in 70% ethanol and dissolve the air-dried pellet into 50 µl sterile distilled water. From that, 40 µl (approximately 1 µg) can be used for restriction analysis in a 50 µl reaction, and 1 µl can be used as template for PCR analysis or for transformation into electrocompetent SW102 bacteria.

3.2 Electroporation of BAC DNA into *E. coli* SW102 strain

The first step of BAC manipulation is to introduce the BAC into SW102 cells that harbor the defective λ prophage and have deletion of the galactokinase (*galK*) gene.

3.2.1 Preparation of Electrocompetent cells—1. Inoculate 5 ml overnight culture in “superbroth special” with an isolated colony of SW102. Grow the culture at 32°C overnight. Use of antibiotics is optional (SW102 cells are resistant to tetracycline and BACs are chloramphenicol resistant).

2. Next morning, dilute the o/n culture 1:50 i.e. transfer 1 ml into an autoclaved 250 ml Erlenmeyer baffled flask with 50 ml “superbroth special” and grow at 32°C to an O.D₆₀₀ 0.50–0.60. Place a 50 ml tube containing sterile ddH₂O in the ice/water slurry.

3. After cooling down the flask containing the bacteria in an ice/waterbath slurry for one or two minutes, transfer 10 ml of culture into pre-cooled Oak Ridge tube and spin down at 6000 g in prechilled rotor for 10 min at 1°C.

4. Discard the supernatant and invert the tube on a paper towel. Add 1 ml cold ddH₂O while keeping the tube in the ice water. Resuspend the pellet in the ddH₂O by gently shaking the tube in the ice/waterbath (this can take a while the first time, around 5 minutes). When resuspended, fill up to 10 ml with ice cold ddH₂O, invert a couple of times and spin again for 5 minutes.

6. Pour off supernatant and resuspend the pellet in 1 ml cold ddH₂O (resuspension will be easy this time) and transfer to a chilled 1.5 ml tube. Spin at 12,000 g for 30s at 1°C.

7. Wash the cells one more time with 1 ml ice cold water.

8. Gently remove all supernatant by inverting the tube on paper towel (be careful not to lose the pellet). Resuspend the cell pellet in 50 µl ice-cold water and keep on ice.

3.2.2 Electroporation—1. Mix 500 ng of BAC DNA (1–5 µl volume) with 50 µl freshly prepared electrocompetent SW102 cells in a pre-cooled 1.5 ml tube. Let it sit for 5 min. on ice and then transfer into a pre-cooled 0.1 cm cuvette.

2. Electroporate the BAC DNA (1.8 kV, 25 µF capacitance and 200 Ω resistance) in cells. Add 1 ml SOC medium immediately after electroporation and transfer the cells into a 1.5 ml tube.

3. Grow the cells for 1 hr at 32°C. Spin down cell for 30s in a microcentrifuge. Discard the supernatant and resuspend the pellet in 200 µl LB medium.

4. Plate the transformed bacteria in a 10 cm LB plate containing 12.5 µg/ml Chloramphenicol. Incubate at 32°C for 18–24 hours. Ten to hundred chloramphenicol resistant colonies may be obtained.

3.2.3 Identification of SW102 clones containing BAC DNA—Isolate the BAC DNA from 8–10 individual BAC colonies using alkaline lysis method as described in section 3.1.1 and perform restriction digestion to confirm the integrity of BAC DNA after electroporation into SW102 cells. Compare the restriction pattern with the original BAC DNA. Most of the BAC DNA from SW102 cells should be identical to the original DNA. Freeze the aliquot of the SW102 cells containing BAC in 15 % glycerol at –70°C.

3.3 Manipulation of BAC DNA using positive-negative selection

Modifications like single base changes or deletions or insertions in the BAC DNA can be generated by using *galK* based positive-negative selection [10]. To perform those steps the first step is to design the primers with the homology arms to generate the targeting vectors.

3.3.1 Generating the Targeting vector for step I—1. Design the first set of primers to amplify the *galK* gene. These primers will have 50–70 bp homology to an area flanking the desired site to be mutated. The 3′ end of these primers anneal to the *galK* cassette (fig 1A). For example, to generate a single base change, the homology arms should be the 50–70 bases on either side of this nucleotide. This will result in a deletion of that nucleotide and insertion of the *galK* gene in the first step. The primers should be as follows:

Forward: 5′–70 bp homology-CCTGTTGACAATTAATCATCGGCA-3′

Reverse: 5′–70 bp homology of complementary strand-TCAGCACTGTCCTGCTCCTT3′

2. Amplify the *galK* cassette using the primers from step 1 and a Taq polymerase with proof reading activity (for example Expand high fidelity Taq from Roche). Use 1–2 ng of *pGalK* plasmid as template. PCR steps are 94°C for 30 sec., 55°C 30 sec., 72°C 1.5 min., for 35 cycles. After the PCR, add 1–2 µl *DpnI* per 50 µl reaction and incubate at 37°C for 1 h. This step removes any plasmid template; *DpnI* digests methylated plasmid but not the PCR products that are not methylated. Gel-purify the *DpnI*-digested PCR product, preferably overnight at low voltage. Extract the DNA from agarose gel using a PCR purification kit and elute in 20 µl ddH₂O (See Note 1).

3.3.2 Induction of bacteriophage λ recombination system in SW102 cells—1.

Start an overnight culture of SW102 cells containing the BAC from a single colony and grow at 32°C in “superbroth special” containing chloramphenicol (12.5 µg/ml).

2. Next morning, dilute 1 ml of overnight culture in 50 ml “superbroth special” containing chloramphenicol (12.5 µg/ml) in a 250 ml baffled conical flask and grow at 32°C to an O.D₆₀₀ of 0.55–0.6. This will take 3–4 hours. During that time turn on the 42°C shaking water bath and make an ice/water slurry. Chill a 50 ml tube containing sterile ddH₂O in ice/water slurry.

3. Once the SW102 culture reaches the O.D₆₀₀ of 0.55–0.6, transfer 10 ml of culture to an Oak Ridge tube and place on ice. This will be the uninduced control. Transfer another 10 ml of culture to another baffled 50 ml conical flask. Heat-shock at 42°C for exactly 15 min. in a shaking water bath. Stop the induction immediately by placing the flask into ice/water slurry for 15 min. with intermittent shaking.

3.3.3 Targeting the *galK* cassette—1. Prepare the electrocompetent cells from both induced and uninduced cells as described in the section 3.2.1 (steps 3 to 7).

¹ Removal of the plasmid DNA is essential to reduce the background *galK*⁺ colonies. Efficiency of incorporation of plasmid DNA is much higher compared to the homologous recombination efficiency and the presence of even picogram quantity of plasmid DNA will give thousands of non-recombinant colonies.

2. Electroporate 300 ng of the targeting vector containing the *galK* cassette (from **3.3.1**) in both uninduced and induced electrocompetent cells as described in the section 3.2.2 (steps 1 and 2).

3. After the 1 hour growth at 32°C, spin down the bacteria in 1.5 ml tube at 12,000 g for 30 sec. and remove the supernatant with a pipette. Resuspend the pellet in 1 ml M9 salts, and spin again. This washing step is repeated once more.

4. After the second wash, discard the supernatant and resuspend the pellet in 1 ml M9 salts. Plate serial dilutions in M9 (100 µl, 100 µl of a 1:10 dilution, and 100 µl 1:100) onto M63 containing biotin and leucine minimal media plates with galactose as the carbon source to select the *galK*⁺ colonies (See Note 2).

5. Incubate the plates for 3 days at 32°C.

3.3.4 Identifying *galK* positive recombinants—1. To screen for *Gal*⁺ colonies, streak 8–10 colonies from above (section 3.3.3) onto MacConkey indicator plates containing galactose and chloramphenicol to obtain single colonies. Incubate the plates at 32°C overnight (See Note 3).

2. Pick 8–10 single red colonies to perform colony PCR to confirm the integration of *galK* cassette at the desired site. Use the primers flanking the targeted region (but not included in the homology arms of the targeting vector) to amplify BAC DNA. The size of the PCR product should show the presence of *galK* cassette into the desired site (See Note 4).

3. Grow the correct clones in 5 ml of “superbroth special” media containing chloramphenicol at 32°C overnight. Use an aliquot of the culture to freeze in 15% glycerol at –70°C and one correct clones to replace the *galK* cassette with desired mutation.

3.3.5 Replacing the *galK* cassette with desired mutation (Step II)—1. Design the PCR primers to generate the targeting vector II that will be used to replace the *galK* cassette with the desired mutation. For this, each primer should have 80 bp homology at the 5′ end and the bases need to be inserted/replaced at the 3′ end (fig. 1B). The two oligonucleotides should have 20 bp complementary bases at the 3′ end that will help to anneal each other and extend the two by PCR to generate a 180 bp targeting vector (fig 1B).

² Prior to selection on minimal media washing in M9 salts is important to remove any rich media from the bacteria. The uninduced samples routinely have a higher degree of lysis/bacterial death after electroporation and some bacteria will be lost, so the uninduced sample is diluted in 0.25 – 0.75 ml M9 salts in the final step to make up for the difference. Plate 100 ml of the uninduced sample as a control.

³ The colonies that appear after the 3 days of incubation are likely to be *Gal*⁺, but they are often mixed with non-recombinant *galK*[–] cells. In order to get rid of any *gal*[–] contaminants, it is important to obtain single, bright red colonies before proceeding to the second step. On the MacConkey agar plates, *galK*[–] colonies will be white/colorless and the *Gal*⁺ colonies will be bright red due to pH change resulting from fermented galactose. Streak the SW102 cells containing the BAC of interest for comparison.

⁴ It is important to check the integrity of the BAC by examining the restriction digestion pattern. Occasionally BACs undergo large deletions. Such BAC clones should be discarded. BACs that show a restriction digestion pattern very similar (a few fragments may be different due to insertion of *galK* cassette) to the parental BAC clone should be used for the next step.

2. Amplify the targeting vector to replace the *galK* cassette using the primers from step 1 and a Taq polymerase with proof reading activity. PCR steps are 94°C 15 sec., 55°C 20 sec., 72°C 20 sec., for 30 cycles. Purify the PCR products using PCR purification kit (Qiagen).
3. Follow the steps described in sections 3.3.2 and 3.3.3 for induction of recombination system and electroporating the targeting vector. After the second wash, discard the supernatant and resuspend the pellet in 1 ml M9 salts. Make serial dilutions in M9 and plate (100 µl, 100 µl of a 1:10 dilution, and 100 µl 1:100) onto M63 containing biotin and leucine minimal containing 2-deoxy-galactose (DOG) and chloramphenicol (12.5 µg/ml) plates with glycerol as a carbon source to select *galK*⁻ colonies.
4. Incubate the plates for 3 days at 32°C.
5. Screen for the correct clones for the replacement of *galK* cassette by colony PCR using the same primers from step 2 of the section 3.3.4. The size of the PCR band should show the absence of *galK* cassette at the desired site. Purify the PCR product and sequence using the same flanking primers in two separate reactions to confirm the presence of the desired mutation (See note 5).
6. Confirm the integrity of the BAC by examining the restriction digestion pattern of BAC miniprep DNA (see section 3.3.1). Discard clones with rearrangements that may have occurred during the BAC manipulation. Include the parent BAC clone as a control. Select the clones that show a digestion pattern identical to the parental clone.

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REFERENCES

1. Muylers JP, Zhang Y, Testa G, Stewart AF (1999): Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* 27:1555–7. [PubMed: 10037821]
2. Zhang Y, Buchholz F, Muylers JP, Stewart AF (1998): A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat Genet* 20:123–8. [PubMed: 9771703]
3. Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL (2000): An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci U S A* 97:5978–83. [PubMed: 10811905]
4. Copeland NG, Jenkins NA, Court DL (2001): Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* 2:769–79. [PubMed: 11584293]
5. Ellis HM, Yu D, DiTizio T, Court DL (2001): High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc Natl Acad Sci U S A* 98:6742–6. [PubMed: 11381128]
6. Sarov M, Schneider S, Pozniakovski A, Roguev A, Ernst S, Zhang Y, Hyman AA, Stewart AF (2006): A recombineering pipeline for functional genomics applied to *Caenorhabditis elegans*. *Nat Methods* 3:839–44. [PubMed: 16990816]

⁵ Rarely, 70–80 bases of homology is not sufficient to obtain correctly targeted clones. In such cases, increasing the length of homology by using an additional set of 100-mer oligonucleotides with 20 bases of homology to the first set of primers is helpful.

7. Swaminathan S, Ellis HM, Waters LS, Yu D, Lee EC, Court DL, Sharan SK (2001): Rapid engineering of bacterial artificial chromosomes using oligonucleotides. *Genesis* 29:14–21. [PubMed: 11135458]
8. Yang Y, Sharan SK (2003): A simple two-step, ‘hit and fix’ method to generate subtle mutations in BACs using short denatured PCR fragments. *Nucleic Acids Res* 31:e80. [PubMed: 12888532]
9. O’Connor M, Peifer M, Bender W (1989): Construction of large DNA segments in *Escherichia coli*. *Science* 244:1307–12. [PubMed: 2660262]
10. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG (2005): Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33:e36. [PubMed: 15731329]
11. Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG (2001): A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73:56–65. [PubMed: 11352566]

Figure 1. Schematic representation of *galK* based recombineering to generate point mutations

A. Generation of targeting vector by PCR to insert the *galK* cassette using primers P1 and P2. 3' end of each P1 and P2 anneal to the 5' and 3' ends of the *galK* cassette, respectively. The 5' ends of the primers have 70 bases of homology to the target site.

B. The targeting vector to replace the *galK* cassette with desired mutation (marked in bold in the box) is generated by PCR using two 100-mer oligonucleotides, P3 and P4. The two oligonucleotides have complementary 3' ends where they anneal together and amplify the homology arms (HA) to generate the targeting vector.

C. The two steps depicting the recombineering procedure to generate point mutation. The base to be replaced in the wild-type BAC and the mutated base in mutant BAC is marked in bold letter in the box. P5 and P6 mark the two PCR primers located outside the homology arms that can be used for screening the correctly targeted clones in both steps of recombineering.