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Using Recombineering to Generate Point Mutations: Oligonucleotide-based “Hit & Fix” Method

Suhwan Chang,

Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702, U.S.A.

Stacey Stauffer,

Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702, U.S.A.

Shyam K. Sharan*

Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702, U.S.A.

Abstract

Ability to manipulate the genome or design genes with desired mutation is critical for functional studies. Recombineering has made genetic manipulation of large genomic fragments very feasible and efficient. In the bacteriophage lambda-based recombineering system, three prophage genes, *exo*, *bet*, and *gam* under the control of a temperature sensitive cI-repressor provide the recombination function. The high efficiency of recombineering by oligonucleotides allows generation of subtle alterations in the bacterial chromosomal DNA as well as episomal DNA. We describe here a two-step “Hit & Fix” method in which a short heterologous sequence is inserted to the target site first (Hit) and this sequence is replaced with the desired mutation in the second step (Fix). Insertion and replacement of the heterologous sequence allows screening of the recombinant clones by PCR or colony hybridization.

Keywords

Recombineering; “Hit & Fix” method; oligonucleotide; bacterial artificial chromosome (BAC); point mutation

1. Introduction

Recombineering is rapidly becoming a standard technology for genetic engineering (1–4). It can be used as an efficient method to generate site-specific mutations in bacterial artificial chromosomes for functional studies. Many different recombineering systems have been described (2,3,5). The method described here utilizes the bacteriophage lambda Red recombination system (2). The bacteriophage lambda genes needed for recombineering are

*Corresponding author: Mailing address: Building 560, Room 32-31C, 1050 Boyles Street, NCI-Frederick, Frederick, MD 21702, USA. Phone: (301) 846-5140, Fax: (301) 846-7017, sharans@mail.nih.gov.

exo, *bet*, and *gam*. The *exo* gene product has 5′–3′ exonuclease activity and the *bet* gene product is a single strand DNA binding protein that promotes annealing. The *gam* gene product inhibits the recBCD nuclease thus preventing the degradation of linear DNA fragments. The minimum homology required for recombination is about 35 bases (2). An *Escherichia coli* strain, Dy380 and its derivative SW102, harboring a defective lambda prophage has been developed such that it promotes much higher recombination efficiencies, as described in the previous chapter (2,6). In these bacterial strains, the prophage provides the recombination genes *exo*, *bet*, and *gam* that is under the control of a temperature sensitive cI-repressor. Therefore, these genes are switched on by inactivation of the repressor by transiently shifting the culture from 32°C to 42°C.

Dy380 and its derivative SW102, have been successfully used to modify exogenous DNA cloned into a BAC vector. However, occasionally, it can be difficult to transfer some BACs into these cells. To overcome this hurdle, a more versatile phage system was sought that would generate high yields of recombinants but at the same time could be easily moved into strains that contain the BAC clones. A few such mobile recombineering systems have been generated such as the mini-lambda (mini-λ), the pSIM plasmid systems and the replication-defective λ phage, λTetR (7,8,9). In this chapter, we describe the use of the mini-λ circular DNA for BAC engineering.

In the mini-λ, all replication and lysis genes have been deleted, creating a replication defective, smaller version of the lambda prophage (12.5kb). The mini-λ provides an inducible Red recombination system that can be introduced by electroporation into nearly any *E. coli* strain, including the *recA* mutant DH10B and its derivatives carrying BACs (7). The mini-λ also contains an antibiotic resistance gene (*e.g.*, tetracycline, kanamycin) as a selection marker. Unlike the lambda prophage in DY380 or SW102 cells, the attachment sites *attL* and *attR* are present in the mini-λ. Consequently, inactivation of the repressor at restrictive temperature activates *int* and *xis* gene expression, causing site-specific excision of the prophage DNA circle. Thus the cells can be cured of the mini-λ DNA and do not remain temperature sensitive. The excision of the mini-λ DNA from the chromosomal DNA also allows purification of the DNA circles from bacterial cells using standard plasmid purification protocols. The mini-λ therefore provides a tractable system to transiently express the phage recombination genes in bacterial cells.

In this chapter, we describe the generation of point mutations without the use of any selection maker (*e.g.* *galK*, as described in the previous chapter). This approach is particularly helpful when multi-copy episomal DNA is being manipulated where selection-counter selection method is not feasible. To generate point mutations without the use of any selectable marker by recombineering, we describe the use of 180-mer oligonucleotides in a two-step “Hit and Fix” method (10). This method is based on the high efficiency of recombineering mediated by single stranded oligonucleotides (11, 12). The two step “Hit & Fix” method was developed to facilitate the screening of recombinant clones (Figure 1A). This is achieved by the introduction of 20 unique nucleotides (unique heterologous sequence) to the site where a mutation has to be generated in the first “Hit” step. In the second “Fix” step, the original sequence is restored with the exception of the point mutation. In both steps, 180-bases-long single-stranded DNA fragments generated by PCR are used as

targeting vectors (Figure 1B). The 20-nucleotide sequence can be used for initial screening by PCR or colony hybridization. Here, we describe the use of the hybridization approach because it allows screening of more than 5,000 individual colonies from a single 15 cm agar plate (13). It also allows simultaneous screening of multiple mutations (in “Hit” step) by using the same hybridization probe. In addition, the heterologous sequence is designed to contain several restriction sites that facilitate rapid confirmation of the correct targeting. 180-mer single stranded oligonucleotides that are used as targeting vectors are generated by PCR using two 100-mer oligonucleotides with 20 base complementary sequences at their 3' end, followed by denaturation of the PCR product.

2. Materials

2.1. Bacterial strain

E. coli DH10B strain containing the bacteriophage mini- λ DNA. This strain is tetracycline resistant. It is temperature sensitive and must be grown at 32°C.

E. coli DH10B containing the BAC clone of interest.

2.2. Equipment

Standard laboratory equipments are used, some of which are described below:

An incubator set at 32 °C, A shaking incubator set at 32 °C and a shaking water bath (200 rpm) set at 42 °C.

Electroporator (Genepulser II with Pulse Controller II, BioRad) and cuvettes with 0.1 gap (BioRad).

High speed centrifuge and a refrigerated microcentrifuge.

Thermal cyclers and accessories for PCR (MyCycler, BioRad)

Spectrophotometer (Beckman-Coulter)

Hybridization oven (VWR)

UV crosslinker 1800 (Stratagene)

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)

2.3. Other reagents

Plasmid DNA purification reagents (Qiagen).

Gel extraction kit or reagents to purify DNA from Agarose gel (Qiagen).

Expand High Fidelity (HiFi) PCR System (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 s

Tetracycline (Sigma)

Chloramphenicol (Sigma)

T4 polynucleotide kinase and buffer (New England Biolabs)

Quick Spin Sephadex G25 Columns (Roche)

LB (Luria Broth) Agar Plate: For 1 liter broth 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl. pH 7.2, 15g agar.

“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 MgCl₂, 10ml of 1M MgSO₄, 20ml of 1M glucose

3. Methods

3.1. Preparation of mini-λ DNA

1. Pick an isolated colony of *E. coli* DH10B containing mini- λ DNA and inoculate 125 ml of “Superbroth special” media containing tetracycline (12.5μg/ml) in a one-liter flask. Culture overnight in a shaking incubator at 32°C.
2. Next morning, induce excision of mini- λ DNA circles by culturing at 42°C for 15 minutes in a shaking water bath.
3. Chill the culture on ice/water slurry for 15 minutes, with occasional shaking.
4. Extract the mini- λ DNA from the cells using a plasmid purification kit.
5. Resuspend DNA pellet in 50 μl of 1X TE buffer (See Note 1).

3.2. Preparation of electro-competent DH10B cells containing desired BAC

1. Pick an isolated colony of DH10B cells containing the desired BAC clone and grow overnight in 3 ml of “Superbroth special” media at 32°C.
2. Next morning, transfer 1ml of the overnight culture to 50 ml of “superbroth special” medium in a 250 ml flask and grow at 32°C to an OD₆₀₀ of 0.55 – 0.60. Transfer 10 ml of the culture to a 50 ml Oak Ridge tube and centrifuge at 6000 x g in a pre-chilled rotor for 10 minutes at 1°C.
3. Gently discard the supernatant and wash the cells twice with 25 ml of ice-cold, sterilized water followed by centrifugation at 6000 x g in a pre-chilled rotor for 10 minutes at 1°C. Carefully remove the supernatant using a pipette and resuspend the pellet in 1 ml of water and transfer to a chilled 1.5 ml tube. Centrifuge at 18,000 x g for 20–30 seconds at 1°C (See Note 2).
4. Wash the cells two more times with 1 ml of ice cold water. Resuspend the cell pellet in water to a total volume of 50µl and keep on ice.

3.3. Electroporation of mini-λ DNA into DH10B cells containing the BAC

1. Mix 1 µl of mini-λ DNA (25–50 ng) with 50 µl of electro-competent DH10B cells containing the BAC and keep on ice for 5 minutes
2. Transfer into a 0.1 cm gap pre-chilled cuvette. Set the Gene Pulser at 1.8 kV, 25 µF capacitance and 200 ohm resistance. Electroporate the DNA into the *E.coli* and immediately add 1 ml of SOC medium. Transfer the cells into a 15 ml falcon tube.
3. Grow cells at 32°C for 1 hour. Spin down the cells and resuspend in 200µl of “superbroth special” medium.
4. Plate the cells on an LB agar plate containing tetracycline (12.5 µg/ml) and incubate overnight at 32 °C.
5. Pick isolated colonies for recombineering. Make glycerol stocks and freeze at –80°C for future use.

3.4. “Hit” step: insertion of a 20-mer heterologous sequence at the site where the point mutation needs to be generated

3.4.1. Generation of a targeting vector for the “Hit” step—A targeting vector for the “Hit” step is generated to insert 20 bases of heterologous sequence in the BAC at the site where a point mutation needs to be generated. The targeting vector is synthesized by PCR using two 100-mer oligonucleotides with overlapping 20 nucleotides at their 3' ends. The resulting targeting vector contains two homology arms that are 80 bases in length, flanking a 20-mer heterologous sequence in the middle (Figure 1B). This heterologous sequence (5'-GGATCCTAGAAATTCCTCGAG-3') can be the same for all “Hit” targeting vectors. This heterologous sequence contains multiple restriction sites that can be used to confirm correct targeting, by restriction enzyme digestion of the DNA amplified from the targeted region.

1. Set up the following reaction using the Expand High Fidelity (HiFi) PCR System (Roche). Use 6 μ l of each 100-mer oligonucleotide (10 μ M) and 10 μ l of 2 mM dNTPs, 2 μ l of HiFi Taq Polymerase (3.5 U/ μ l) in a 100 μ l PCR reaction. PCR cycle includes an initial denaturation at 94°C for 1 minute followed by 30 cycles of 94°C for 30 sec, 55–60°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 2 minutes.
2. Examine 1 μ l of the reaction on a 1.0 –1.5 % agarose gel in 1xTAE buffer to confirm the amplification of an 180bp PCR product.
3. Purify the targeting vector using a Qiagen PCR Purification Kit and elute in 30 μ l of Qiagen Elution Buffer or ethanol-precipitate and dissolve in 20–30 μ l dH₂O.
4. Denature the PCR product by heating at 94°C for 5 minutes and immediately chill on ice to obtain single stranded 180-mer oligonucleotides.

3.5. Induction of the lambda recombination genes and preparation of electro-competent cells

1. Inoculate DH10B cells containing the BAC and the mini- λ from an isolated single colony into 3 ml “superbroth special” medium. Culture overnight at 32°C, in a shaking incubator.
2. Add 1 ml of the overnight culture to 30 ml of “superbroth special” medium in a 250-ml baffled Erlenmeyer flask.
3. Grow cells at the 32°C until the OD₆₀₀ is between 0.55 and 0.6.
4. Transfer 10 ml of the culture in a 50 ml Oak Ridge tube and store on ice. These cells will be used as uninduced control (See Note 3).
5. Transfer 10 ml of the culture in to a 50 ml Erlenmeyer flask and place in a 42 °C shaking water bath. Shake for 15 min at 200 rpm to induce the lambda recombination genes.
6. Immediately chill the flask on ice/water slurry with gentle swirling for 10–15 min.
7. Transfer the culture into a pre-chilled 50 ml Oak Ridge tube.
8. Centrifuge both the induced and uninduced cultures for 10 min at 4600 x *g* at 4 °C.
9. Make cells electro-competent as described in section 3.2.

3.6. Electroporation of the targeting vector into DH10B cells containing the BAC

1. Chill two 0.1-cm electroporation cuvettes in a freezer.
2. In a 0.5 ml tube, mix appropriate volume of DNA (200 to 300 ng of salt-free PCR fragment) with 50 μ l of electro-competent induced or uninduced cells. Leave the tubes on ice for 5 minutes.

3. Electroporate the DNA into the cells using 1.8 kV, 25 μ F capacitance and 200 ohm resistance. Immediately add 1 ml SOC medium to the cuvette and transfer the electroporation mix to sterile, 15ml culture tubes. Incubate the tubes with shaking at 32 °C for 1.5 hr.
4. Serially dilute the cell suspension in “superbroth special” medium and plate 200 μ l of 10^{-3} and 10^{-4} dilutions onto a 15 cm LB agar plate containing an appropriate antibiotic (Chloramphenicol at 12 μ g/ml). Use sterile glass beads instead of a bacteriological spreader to achieve uniform distribution of colonies throughout the plate. Incubate agar plates for 18–22 h at 32 °C.

3.7. Identifying the recombinant clones by colony hybridization

1. Pick the plate with approximately 3000–6000 colonies. Avoid using plates with too few or too many colonies.
2. Cover the plate with a circular, charged nylon membrane (Hybond) and mark three edges by punching holes with a 17-gage needle.
3. Carefully lift up the membrane with two forceps. Autoclave the membrane for 1 minute in dry cycle to denature the bacterial DNA. Cross-link DNA on the membrane by using a UV crosslinker and perform colony hybridization.
4. Generate γ - 32 P-labeled probe using a 20-mer oligonucleotide complementary to the heterologous sequence. Set up the labeling reaction in a 20 μ l volume by mixing 2 μ l of 10X Polynucleotide Kinase buffer, 1 μ l of 10 μ M oligonucleotide, 5 μ l of [γ - 32 P] ATP (5000 Ci/mmol, 10 mCi/ml, Amersham), 1 μ l of 10 U/ μ l T4 polynucleotide kinase. Incubate at 37°C for 45 min.
5. Inactivate the enzyme at 68 °C or by boiling for 1 min. Add 30 μ l TE to bring the total volume of reaction mix up to 50 μ l.
6. Purify the labeled oligonucleotides using Sephadex G-25 columns (Quick Spin Columns, Roche). Measure the activity 1 μ l of the product in a scintillation counter. The typical activity is usually around 100,000–200,000 cpm. Use one third of the labeled probe for the hybridization. The rest can be stored at –20°C and used for the subcloning step (see below)
7. Hybridize for 2–3 hours at 50 °C. Hybridizing for more than 3 hours will result in high background. Wash and expose to X-Ray film to identify positive colonies.
8. After positive colonies are identified, they should be subcloned to obtain a pure recombinant clone that does not contain the original non-recombinant BAC by a second round of hybridization. Repeat steps 2–7 of section 3.7.
9. Confirm correct targeting by testing for the presence of the restriction sites present in the heterologous sequence (See Note 4).
10. Select the recombinant clone for presence of the mini- λ by streaking the colonies on LB tet plate. Colonies that are tetracycline resistant have retained the mini- λ and can be used for next “Fix” step (See Note 5).

“Fix” step: Replace the heterologous sequence with the desired mutation

To replace the heterologous sequence inserted in the “Hit” BAC DNA with a sequence having the desired mutation, a second round of recombineering will be performed. “Fix” targeting cassette is composed of the same homology arms as the corresponding “Hit” vector, while the heterologous middle region of the “Hit” vector is replaced with the final sequence including a desired mutation. At this point, a 20-mer oligonucleotide encompassing this region can serve as a probe to differentiate the “Fix”- recombinants from the “Hit”-intermediate clones (See Note 6).

1. Once the correctly targeted pure “Hit” colonies are identified, repeat the targeting step (Fix targeting) to replace the 20-nucleotide heterologous sequence with the desired mutation exactly as described above in sections 3.4. – 3.7.
2. Check the integrity of the BAC DNA by digesting the BAC with a few restriction enzymes (*e.g. BamHI, EcoRI, HindIII, EcoRV*) and comparing the restriction pattern with the original BAC clone by running the two samples in parallel on a 0.8 % agarose gel.
3. Confirm the mutation by sequencing. Make a glycerol stock and freeze at –80 °C.

4. NOTES

1. The yield of mini-λ DNA is considerably lower compared to the yield of high copy plasmids. Resuspending the DNA pellet in 30–50μl ensures that the DNA is not too dilute. DNA concentration of 25–50 ng/μl is desirable.
2. Remove tubes from the centrifuge promptly. Because the pellet is very soft, care should be taken not to dislodge it, especially when processing multiple tubes.
3. It is very important to have a negative control when screening for recombinant clones. Uninduced control should not yield any positive clone. Signals on the autoradiograph from uninduced cells should be considered as background signal.
4. To confirm the presence of restriction sites, use two PCR primers outside the homology arms of the targeting vector. Digest the PCR product for the presence of *BamHI*, *EcoRI* or *XhoI* sites present in the heterologous sequence.
5. Because the attachment sites *attL* and *attR* are present in mini-λ DNA, the prophage DNA circle can be excised out and either it can reintegrate or be lost from the cell during heat induction. If no tetracycline resistant colony is obtained, the mini-λ DNA should be electroporated in one of the “Hit” clones as described in sections 3.2. and 3.3.
6. Although a 20-mer oligonucleotide works well as a probe, occasionally, a longer oligonucleotide gives more specific signal. If the 20-mer oligonucleotide probe either results in very weak signal or very high background signal, 35–40-mer oligonucleotide should be tried.

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Figure 1. Schematic representation of the “Hit & Fix” method to generate subtle mutations using single stranded oligonucleotides as targeting vector.

A. Scheme to generate subtle alterations (*e.g.* T to G) without the use of a selectable marker using 180-mer oligonucleotides by a the two step “Hit and Fix” method. In step 1, a 180-mer single-strand oligonucleotide is used to replace 20 nucleotides (gray box) around the target site with 20 bases of a heterologous sequence (black box). Recombinants can be identified by colony hybridization using an end-labeled 20-mer oligonucleotide. Generation of a correct recombinant clone can be confirmed by digesting the PCR product (~300–500 bp) of primers P1 and P2 with *Bam*HI, *Eco*RV or *Xho*I (recognition sequence present in the heterologous sequence). In step 2, the 20 nucleotides are restored to the original sequence, except for the desired mutation. Such clones can be identified by colony hybridization using a 20-mer oligonucleotide as probe. The generation of correct recombinant clones can be

tested by the loss of the restrictions sites by digesting the PCR product of primers P1 and P2, and confirmed by sequencing.

B. The single-stranded oligonucleotides containing 80 bases of 5' and 3' homologies and 20 bases of a heterologous sequence (contains restriction sites *Bam*HI, *Eco*RV, *Xho*I) can be generated by using two 100-mer oligonucleotides as forward and reverse primers in a PCR reaction. The two 100-mer oligonucleotides have 20 complementary bases at the 3' end. To obtain single stranded oligonucleotides, the 180 bp PCR product can be denatured at 94°C for 5 minutes and chilled on ice.