Markerless and Marker-Exchange Mutagenesis

(1) Amplify and purify arms A and B of target gene

1. Following the primer design protocol, design two sets of primers for the regions flanking the deletion target region, to be named arms A and B. *
2. Amplify arms A and B using these primers and high-fidelity polymerase via PCR following the manufacturer's instructions.
3. Run the PCR products through 1% (w/v) agarose gel (tris-acetate-EDTA buffer) via electrophoresis. Excise the bands corresponding to arms A and B, then extract and purify DNA from the gel using a kit of choice. (QIAquick Gel Extraction Kit is used.)

* When designing primers, make sure the amplicons do not contain restriction sites except for those at either end to prevent incorrectly truncated arms. For directional insertion into the vector, use 3 distinct, compatible restriction sites, one on either end of the arms AB construct, and identical site connecting arms A and B. For an example of the construct, refer to Figure 1. (Online compatibility check for NEB restriction enzymes: https://nebcloner.neb.com/#!/redigest)

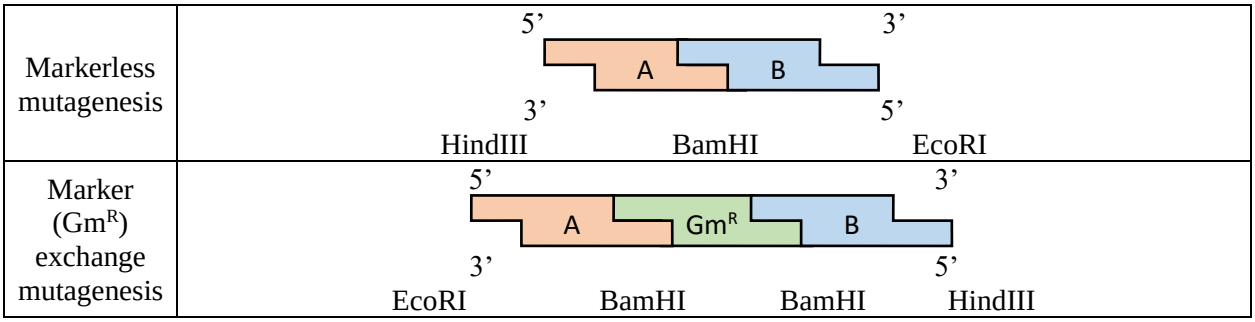

Figure 1. Construction of inserts

(2) Construct recombinant vector (with Gm^R cassette)

Vector preparation:

1. Digest *pK18mobsacB* with restriction enzymes (*HindIII* and *EcoRI* from example in Figure 1) following the manufacturer's instructions.
2. If applicable, heat inactivate the restriction enzymes, then purify DNA using a kit of choice. (QIAquick PCR Purification Kit is used.)

Insert preparation:

1. Digest arms A and B with respective restriction enzymes.

2. If applicable, heat inactivate the restriction enzymes, then purify DNA using a kit of choice. (QIAquick PCR Purification Kit is used.)

Ligation:

1. Measure the DNA concentration of *pK18mobsacB*, arm A, and arm B. Then, calculate the required mass of each for a ligation reaction where the insert to vector ratio is at least 3:1. (Online calculator: http://nebiocalculator.neb.com/#!/ligation)
2. Ligate the *pK18mobsacB* plasmid with the arms following manufacturer's instructions. (T4 DNA Ligase from NEB is used.)

E. coli transformation:

1. Transform *E. coli* appropriate for plasmid maintenance using the ligation product following the manufacturer's instructions. (*E. coli* TOP10 is used.)
2. After transformation, spread the *E. coli* suspension on an LB plate supplemented with 25 μg/mL kanamycin for selection. Then, incubate the plate at 37°C overnight.
3. Pick and resuspend colonies that grew overnight in separate PCR tubes in 15 μL of sterile water. Use 5 μL of the suspension to perform PCR to verify correct insertion. Once the transformant is verified via PCR, save the colony by either spotting the remaining 10 μL of suspension on a new LB+kan plate or inoculating fresh LB+kan liquid medium. For additional verification, send the PCR product for sequencing.
4. After all verification, grow 5 – 10 mL of *E. coli* with the correct recombinant vector, then extract the vector using a kit of choice. (QIAprep Spin Miniprep Kit is used.)

For marker exchange mutagenesis:

1. Digest the verified recombinant vector from above with the restriction enzyme targeting the site connecting arms A and B (*BamHI* from example in Figure 1). Make sure to dephosphorylate vector to prevent re-ligation. (Quick CIP from NEB is used.)
2. Purify DNA using a kit of choice. (QIAquick PCR Purification Kit is used.)
3. Digest p34S-Gm plasmid with the same restriction enzyme used in step 1.
4. Run the p34S-Gm digestion products through 1% (w/v) agarose gel (tris-acetate-EDTA buffer) via electrophoresis. Excise the band corresponding to Gm^R (~0.9 kb), then extract and purify DNA from the gel using a kit of choice. (QIAquick Gel Extraction Kit is used.)
5. Ligate the recombinant vector with the Gm^R following the two-step ligation procedure above.

(3) Conjugation and screening for mutants

Conjugation:

1. Transform the conjugation donor *E. coli* S17-1 with the recombinant vector following the manufacturer's instructions.
2. After transformation, spread the *E. coli* suspension on an LB plate supplemented with 25 μg/mL kanamycin for selection. Then, incubate the plate at 37°C overnight.
3. Pick and resuspend colonies that grew overnight in separate PCR tubes in 15 μL of sterile water. Use 5 μL of the suspension to perform PCR to verify correct insertion. Once the transformant is verified via PCR, save the colony by either spotting the remaining 10 μL of suspension on a new LB+kan plate or inoculating fresh LB+kan liquid medium.
4. After all verification, grow 10 mL of *E. coli* with the correct recombinant vector, then proceed to conjugation and screening mutants following the conjugation protocol.