Protein Science - Engineering Cassettes for Scarless Lambda-Red-Mediated Genomic Manipulation

Purpose

The purpose of this document is to provide instructions to generate linear cassettes using the CAT-SacB dual selection marker.

Scope

Similar constructs have been used in our laboratory to facilitate marker-less alterations to both *E. coli* and baculoviral genomes. Here, we have included procedures for cassettes to make both knockouts (Procedures, A) and insertions (Procedures, B), as well as the second-step removal of the CAT-SacB markers (Procedures, C and D).

Definitions

- **PCR:** polymerase chain reaction
- **Tm:** melting temperature

Materials and Equipment:

- 5 µM oligonucleotides (detail in text)
- pELO4 (Dr. Donald Court, NCI at Frederick)
- Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Inc., M0531S)
- Deionized water
- 10× TE (Fisher BioReagents, BP2477-1), diluted to 1× and filter sterilized
- 0.2 mL PCR tubes (USA Scientific, Inc., 1401-8100)
- 10× gel loading dye (100 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 50 mM NaCl, 0.3% xylene cyanol [Sigma-Aldrich, X4126], 3% tartrazine [Sigma-Aldrich, T0388], 50% glycerol), diluted to 2× with deionized water
- 1 kb Plus ladder (New England Biolabs, Inc., N3200L) (100 µL DNA ladder, 20 µL 10× gel loading dye, 350 µL 1× TE, 5 µL 0.5 M EDTA [pH 8.0])
- Agarose gel 0.8% in 1× TAE with ethidium bromide (Embi Tec, GE-3701)
- 10× TAE (Embi Tec, EC-1016), diluted to 1× in deionized water
- QIAquick PCR Purification Kit (QIAGEN, 28106)
- 1.5 mL tubes (USA Scientific, Inc., 1615-5500)
- Gibson Assembly HiFi Kit (Codex DNA, Inc., GA1100-50)
- Thermocycler
- Electrophoresis equipment
- Gel imager with UV light and camera
- Spectrophotometer

Safety Precautions

Ethidium bromide is a mutagen and should be handled with great care. Liquid waste as well as contaminated tips and other disposables must be disposed of properly in accordance with your organization's guidelines.

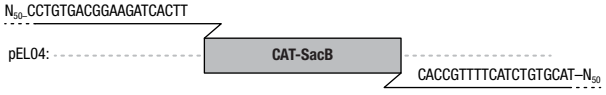
Procedures

A. CAT-SacB Cassette for Knockouts

1. Design the oligonucleotides.

   a. The forward oligonucleotide should include 50 bp of the DNA sequence directly upstream of the region you wish to remove, followed by the pELO4-specific sequence: (5′–3′) N_{50}-CCTGTGACGGAAGATCACTT.

   b. The reverse oligonucleotide will have 50 bp of sequence directly downstream of the region you wish to remove and the following pELO4-specific top-strand sequence: CACCGTTTTCATCTGTGCAT-N_{50}. The reverse oligonucleotide sequence will then need to be reverse-complemented before synthesis.



   c. Both oligonucleotides can be synthesized at whatever concentration you prefer and then diluted to a 5 µM concentration.

2. Amplify the cassette by PCR.

   a. In a 0.2 mL PCR tube, set up a 100 µL PCR reaction including:

      - 20 ng pELO4 pDNA

- 0.2 µM forward oligo
- 0.2 µM reverse oligo
- 1 mM MgCl2
- 50 µL Phusion® High-Fidelity PCR Master Mix
- Deionized water to a final volume of 100 µL

b. Place the tube in the thermocycler and amplify the DNA using the following conditions with the Tm being optimized for the included oligonucleotides: initial denaturation at 98°C for 45 seconds; 25 cycles of 98°C for 30 seconds, 62°C for 30 seconds, and 72°C for 2 minutes; followed by a 10-minute final elongation at 72°C and cooling to 4°C.

c. Set up electrophoresis equipment and fill with 1× TAE. Insert 0.8% agarose gel into the dock.

d. In a fresh 0.2 mL PCR tube, mix 5 µL of the PCR product with 5 µL of 2× loading dye and load the full volume onto a 0.8% agarose gel. Load 1 µL of 1 kb Plus ladder with loading dye in a neighboring well.

e. Electrophorese the DNA samples for 1 hour at 100 V.

f. Image the gel with UV light. The expected amplicon is 3.2 kb.

3. Purify the amplicon.

a. Purify the remaining PCR reaction volume using the QIAquick PCR Purification Kit per the manual instructions.

b. Measure the concentration of the eluted DNA with a spectrophotometer.

c. The final concentration will need to be >25 ng/µL for future electroporation of 100 ng. If necessary, concentrate the DNA by your preferred method, but do not allow it to become overdry or heated at a high temperature.

d. Store the final amplicon at –20°C until ready to transform.

B. CAT-SacB Cassette for Insertions

1. Design the construct.

a. To add a gene(s), analyze the host genome for a region that can tolerate insertions. You will need to include a host-specific promoter, the ORF sequence, and a terminator/polyadenylation signal for the inserted gene to be functional. If you are altering an existing gene, be especially careful in designing the replacement construct to be certain not to introduce unwanted indels.

2. Amplify CAT-SacB.

a. The 3.2 kb dual-selection marker can be amplified without homology using these 5′–3′ oligonucleotide sequences: CAT forward (CCTGTGACGGAAGATCACTT) and SacB reverse (ATGCACAGATGAAAACGGTG).

b. In a 0.2 mL PCR tube, set up a 100 µL PCR reaction including:

- 20 ng pELO4 pDNA
- 0.2 µM forward oligo
- 0.2 µM reverse oligo
- 1 mM MgCl2
- 50 µL Phusion® High-Fidelity PCR Master Mix
- Deionized water to a final volume of 100 µL

c. Place the tube in the thermocycler and amplify it using the following conditions: initial denaturation at 98°C for 45 seconds; 25 cycles of 98°C for 30 seconds, 62°C for 30 seconds, and 72°C for 2 minutes; followed by a 10-minute final elongation at 72°C and cooling to 4°C.

3. Design oligonucleotides for strain-specific pieces.

a. Design forward and reverse oligonucleotides for each component of your intended insertion cassette to include ~20 bp of gene-specific sequence. There must also be ~40 bp of homology between each of the pieces; we typically add the forward oligonucleotide of a given piece to the end of the previous piece as shown in the diagram. Given the CAT-SacB amplicon is tagless, you will need to include homology to the beginning of CAT on the reverse of the previous piece as well as homology to the end of SacB on the forward of the following piece.

4. Amplify the components by PCR.
   a. In a 0.2 mL PCR tube, set up a 100 µL PCR reaction as such:
      - 20 ng genomic DNA, synthetic or other plasmid template
      - 0.2 µM forward oligo
      - 0.2 µM reverse oligo
      - 50 µL Phusion® High-Fidelity PCR Master Mix
      - Deionized water to a final volume of 100 µL
   b. Place the tube in the thermocycler and amplify it using the following conditions with the Tm specific to the oligonucleotide pairs: initial denaturation at 98°C for 45 seconds; 25 cycles of 98°C for 30 seconds, Tm°C for 30 seconds, and 72°C for 30 seconds per kb of the expected product; followed by a 10-minute final elongation at 72°C and cooling to 4°C.
   c. Set up electrophoresis equipment and fill it with 1× TAE. Insert a 0.8% agarose gel into the dock.
   d. In a fresh 0.2 mL PCR tube, mix 5 µL of the PCR product with 5 µL of 2× loading dye and load the full volume onto a 0.8% agarose gel. Load 1 µL of 1 kb Plus ladder with loading dye in a neighboring well.
   e. Electrophorese for 1 hour at 100 V.
   f. Image the gel with UV light. The expected amplicon is 3.2 kb.
5. Purify the amplicons.
   a. Purify the remaining PCR reaction volume using the QIAquick PCR Purification Kit per the manual instructions
   b. Measure the concentration of the eluted DNA with a spectrophotometer.
   c. Based on the size of each piece and the concentration, calculate fmol/µL. (Use the formula ng DNA × fmol/660fg × 106fg/1 μg × 1/# nucleotides = fmol.)
   d. Store the purified amplicons at –20°C until ready to transform.
6. Assemble the CAT-SacB cassette.
   a. Thaw Gibson Assembly HiFi 2× Mastermix on ice.
   b. Add 25 fmoles of each amplicon to a 0.2 mL PCR tube and bring volume up to 10 µL with deionized water.
   c. Add 10 µL Gibson Assembly HiFi 2× Mastermix and place the tube in the 50°C heat block for 30 minutes.
7. Amplify the CAT-SacB cassette.
   a. Add the following to a 0.2 mL PCR tube:
      - 1 µL Gibson Assembly Reaction
      - 0.2 µM forward primer
      - 0.2 µM reverse primer
      - 50 µL Phusion® High-Fidelity PCR Master Mix
      - Deionized water to a final volume of 100 µL
   b. Place the tube in the thermocycler and amplify it using the following conditions with the Tm specific to the oligonucleotide pairs: initial denaturation at 98°C for 45 seconds; 20 cycles of 98°C for 30 seconds, Tm°C for 30 seconds, and 72°C for 30 seconds per kb of the expected product; followed by a 10-minute final elongation at 72°C and cooling to 4°C.
   c. Set up the electrophoresis equipment and fill it with 1× TAE. Insert a 0.8% agarose gel into the dock.
   d. In a fresh 0.2 mL PCR tube, mix 5 µL of the PCR product with 5 µL 2× loading dye and load the full volume onto a 0.8% agarose gel. Load 1 µL of 1 kb plus ladder with loading dye in a neighboring well.
   e. Electrophorese the DNA samples for 1 hour at 100 V.
   f. Image the gel with UV light.
   g. Confirm the size of the amplicon.
8. Purify the CAT-SacB cassette.
   a. Purify the remaining PCR reaction volume using the QIAquick PCR Purification Kit per the manual instructions.
   b. Measure the concentration of the eluted DNA with a spectrophotometer.
   c. The final concentration will need to be >25 ng/µL for future electroporation. If necessary, concentrate the DNA by

your preferred method but do not allow it to become overly dry or heated at a high temperature.

d. Store the final amplicon at –20°C until ready to transform.

C. CAT-SacB dsDNA Removal Cassette

1. Design the oligonucleotides.

a. Design forward and reverse oligonucleotides to amplify a fragment of genomic DNA ~300 bp long directly upstream of the inserted CAT-SacB markers.

b. Design forward and reverse oligonucleotides to amplify a fragment of genomic DNA ~300 bp directly downstream of the inserted CAT-SacB markers. The forward oligonucleotide will also contain a 25 bp flank that has homology added to the end of the upstream homology arm.

c. All oligonucleotides can be synthesized at whatever concentration you prefer and then diluted to 5 µM concentration with 1× TE.

2. Amplify the homology arms by PCR.

a. In a 0.2 mL PCR tube, set up a 100 µL PCR reaction including:

- 200 ng gDNA
- 0.2 µM forward oligo
- 0.2 µM reverse oligo
- 12.5 µL Phusion® High-Fidelity PCR Master Mix
- Deionized water to a final volume of 25 µL

b. Place the tube in the thermocycler and amplify it using the following conditions, adjusting the Tm to the oligonucleotides you have designed: initial denaturation at 98°C for 45 seconds; 25 cycles of 98°C for 30 seconds, Tm°C for 30 seconds, and 72°C for 30 seconds per kb of the expected product; followed by a 10-minute final elongation at 72°C and cooling to 4°C.

c. Set up the electrophoresis equipment and fill with 1× TAE. Insert a 0.8% agarose gel into the dock.

d. In a fresh 0.2 mL PCR tube, mix 5 µL of the PCR product with 5 µL of 2× loading dye and load the full volume onto a 0.8% agarose gel. Load 1 µL of 1 kb Plus ladder with loading dye in a neighboring well.

e. Electrophorese the DNA samples for 1 hour at 100 V.

f. Image the gel with UV light. The expected amplicon is 3.2 kb.

3. Purify the amplicons.

a. Purify the remaining PCR reaction volume using the QIAquick PCR Purification Kit per the manual instructions.

b. Store the final amplicon at –20°C until ready for overlap PCR.

4. Overlap PCR.

a. Combine the following in a 0.2 mL PCR tube:

- 0.2 µL purified upstream homology arm
- 0.2 µL purified downstream homology arm
- 5 µM upstream arm forward oligo
- 5 µM downstream arm reverse oligo
- 50 µL Phusion® High-Fidelity PCR Master Mix
- Deionized water to a final volume of 100 µL

b. Place the tube in the thermocycler and amplify it using the following conditions adjusting the Tm to the oligonucleotides you have designed: initial denaturation at 98°C for 45 seconds; 25 cycles of 98°C for 30 seconds, Tm°C for 30 seconds, and 72°C for 30 seconds per kb of the expected product; followed by a 10-minute final elongation at 72°C and cooling to 4°C.

c. Set up the electrophoresis equipment and fill it with 1× TAE. Insert a 0.8% agarose gel into the dock.

d. In a fresh 0.2 mL PCR tube, mix 5 µL of the PCR product with 5 µL of 2× loading dye and load the full volume onto a 0.8% agarose gel. Load 1 µL of 1 kb Plus ladder with loading dye in a neighboring well.

e. Electrophorese the DNA samples for 1 hour at 100 V.

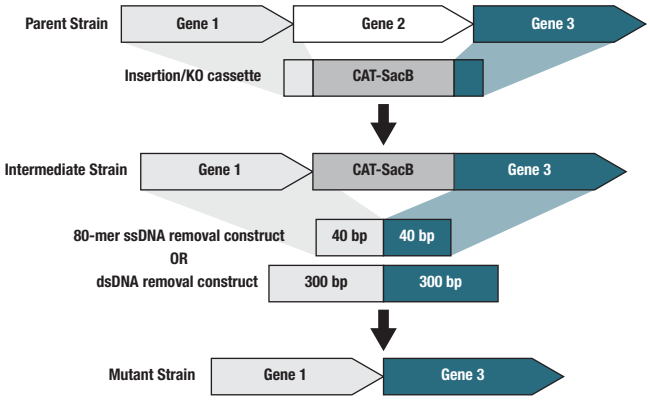
f. Image the gel with UV light. The expected amplicon will be the sum of the two homology arms (~600 bp).

5. Purify the linear CAT-SacB removal cassette.
    a. Purify the remaining PCR reaction volume using the QIAquick PCR Purification Kit per the manual instructions.
    b. Take a concentration of the eluted DNA with a spectrophotometer.
    c. The final concentration will need to be >25 ng/µL for future electroporation of 100 ng. If necessary, concentrate the DNA by your preferred method but do not allow it to become overly dry or exposed to high temperatures.
    d. Store the final amplicon at –20°C until ready to transform.

D. CAT-SacB ssDNA Removal Oligonucleotides

If you are making a deletion, point mutation, or small insert, you may be able to remove the CAT-SacB cassette with a single-stranded DNA. Design this as an 80-mer oligonucleotide with 40 bp of homology to the region directly upstream of the alteration and 40 bp of homology to the region directly downstream of the alteration. The highest recombineering efficiency will be achieved with this being designed on the lagging strand.

Recombination Scheme



References

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