Protein Science - Scarless Lambda-Red Recombineering of Baculovirus

Purpose

The purpose of the procedures in this document is to generate genomic alterations to baculoviral plasmids.

Scope

This protocol can be used to insert/remove regions of baculoviral genomic DNA using cassettes generated in the SOP titled "Engineering Cassettes for Scarless Genomic Manipulation."

Definitions

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

Materials and Equipment

- Intact Bacmid DNA from the strain you wish to alter
- Lambda-Red-competent *E. coli* strain, such as SW106 (Dr. Donald Court, NCI at Frederick)
- Luria broth (LB) agar plate
- LB media (MP Biomedicals, 113002-011)
- 15 mL Falcon tube (VWR, 60819-761)
- Circular bacmid DNA, such as bMON14272 from Bac-to-Bac®
- Insertion cassette or knockout cassette generated in the SOP titled "Engineering Cassettes for Scarless Genomic Manipulations"
- 1.5 mL tubes (USA Scientific, Inc., 1615-5500)
- Electroporation cuvettes (Bio-Rad Laboratories, 1652089)
- 100 mg/mL ampicillin (Sigma-Aldrich, A9518), stock dilution 100 mg/mL in deionized water, filter sterilized
- Kanamycin (Sigma-Aldrich, K1377), stock dilution 50 mg/mL in deionized water, filter sterilized
- Chloramphenicol (Sigma-Aldrich, C1919), stock dilution 30 mg/mL in ethanol, filter sterilized
- Tetracycline (Sigma-Aldrich, T7660), stock solution 12 µg/mL in ethanol, filter sterilized
- LB agar plates with 50 µg/mL kanamycin
- QIAprep Spin MiniPrep Kit (QIAGEN, 27106)
- 10× 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])
- Supercoiled ladder (New England Biolabs, Inc., N0472S), 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% with Ethidium Bromide [Embi Tec, GE-3701])
- Oligonucleotides 5 µM (detail in text)
- Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Inc., M0531S)
- Arabinose 10% w/v (Sigma-Aldrich, A3256), filter sterilized
- 50 mL conical tubes (VWR, 21008-951)
- Deionized water
- 0.2 mL PCR tubes (USA Scientific, Inc., 1402-8100)
- Glycerol (Sigma-Aldrich, G5516) diluted to 60% with deionized water and filter sterilized
- 10× TAE (Embi Tec, EC-1016) diluted to 1× with deionized water
- 50 mL Erlenmeyer flask with baffles
- 5× M9 salts (Sigma-Aldrich, M6030) diluted to 1× in water and filter sterilized
- LB no salt, 6% sucrose agar plates with 100 µg/mL ampicillin
- LB agar plates with 50 µg/mL kanamycin, 12 µg/mL tetracycline
- CAT-SacB Removal Cassette (reference the SOP titled "Engineering Cassettes for Scarless Genomic Manipulations")
- DE96 or similar bacmid-free DH10Bac-derivative strain with resident helper plasmid
- 2 mL tube (USA Scientific, Inc., 1620-2700)
- Plasmid Prep Buffer A: 25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.9% (w/v) d-glucose
  - Store at room temperature.

- Plasmid Prep Buffer B: 0.20 M NaOH, 1.33% (w/v) sodium dodecyl sulfate (SDS)
  - Store at room temperature.
  - SDS may precipitate at low temperatures—redissolve before use by heating at 37°C.
  - Make fresh after 6 months and be sure to cap immediately after use.
- Plasmid Prep Buffer C: 7.5 M ammonium acetate
  - Store at 4°C.
- Bacmid Resuspension Buffer: 10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.1 µg/mL ribonuclease A (Sigma-Aldrich)
  - Store at room temperature for up to 6 months.
- 100% ethanol
- 42°C water bath
- 30°C incubator with shaking platform
- 37°C incubator with shaking platform
- Beckman Coulter floor centrifuge
- Eppendorf benchtop centrifuge
- QIAquick PCR Purification Kit (QIAGEN, 28106)
- Thermocycler
- Electrophoresis equipment
- Gel imager with UV light and camera
- Spectrophotometer
- Electroporator

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.

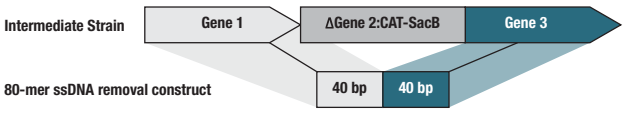
Things to Note

- SW106 contains a temperature-sensitive λ prophage and should not be grown above 32°C. Increased temperature growth may result in undesired recombination events or prophage excision. Induction of lambda-Red enzymes is done at 42°C to remove the C1857 repressor at the intended timepoint, just prior to transforming recombination cassettes.
- Lambda-Red electrocompetent cells should be made fresh on the day of transformation to ensure the lambda-Red proteins will be functional and abundant.
- We have not seen any undesired genomic rearrangements using this method on AcMNPV bacmids and typically now only sequence the region that has been altered, but you can add an additional step of purifying the genomic DNA and submitting it for whole-genome sequencing if you have concerns.

Procedures

A. Oligonucleotide Design

1. Design forward and reverse oligonucleotides for the region being altered. Be certain these bind outside of the homology arms of the recombineering cassette. These confirmation oligonucleotides will be used to verify the insertion and removal of the cassette.
2. If you are making a deletion, point mutation, or fairly 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. See the figure below for an example of a knockout of Gene 2. Most inserts will require the longer removal cassette detailed in the SOP titled "Engineering Cassettes for Scarless Genomic Manipulations."



3. Design oligonucleotides to sequence the region that will be altered. The confirmation oligonucleotides can be used for sequencing the junctions, and then you will need a top-strand oligonucleotide roughly every 500 bp throughout the region to ensure overlapping sequence coverage.

B. Prepare Electrocompetent SW106

1. Streak out SW106 from stab to LB agar plates and grow it at 30°C overnight.
2. Pick a single colony to 2 mL LB media in a 15 mL Falcon tube and grow it overnight at 30°C, shaking at 250 rpm.
3. Make a 15% glycerol stock for this culture and inoculate 10 mL LB with 250 µL of culture in a 50 mL flask.

4. Grow it at 30°C, shaking at 250 rpm, and chill 20 mL deionized water in a 50 mL conical tube on ice.
5. When the culture reaches OD_{600} = 0.5, place it on ice until chilled.
6. Spin down the cells in a 4°C centrifuge for 5 minutes at 5,000 × *g*.
7. Pour off the media to a waste container.
8. Resuspend the pellet in 5 mL chilled deionized water by agitating it on ice.
9. Add additional 10 mL chilled deionized water.
10. Spin the sample in a 4°C centrifuge for 5 minutes at 5,000 × *g*.
11. Pour off the supernatant to a waste container.
12. Resuspend the sample in 1 mL chilled deionized water by gently pipetting it and move it to a 1.5 mL tube.
13. Spin the sample in a benchtop centrifuge for 30 seconds at 13,000 × *g*.
14. Remove the supernatant with a pipette and resuspend the pellet in 50 µL chilled deionized water by gently pipetting it.
15. Place it on ice until ready to transform.

C. Prepare Bacmid Recombineering Strain

1. In a 1.5 mL tube, combine 50 µL of electro-competent SW106 cells of the strain you wish to alter and 1 µL of your preferred circular bacmid DNA on ice.
2. Incubate it on ice for 15 minutes.
3. Carefully move the mixture to the gap in an electroporation cuvette, avoiding bubbles.
4. Electroporate with 1.75 kV and immediately flush with 1 mL LB.
5. Remove the transformants by pipetting the sample into a fresh 15 mL Falcon tube and incubate at 30°C, shaking for 1 hour.
6. Plate 100 µL of the sample on LB agar plates with 50 µg/mL kanamycin and incubate it overnight at 30°C.
7. Add 4 mL LB and 4 µL of 50 mg/mL kanamycin to a 15 mL Falcon tube.
8. Inoculate the media with a single colony from the *SW106-bmon14272* transformation plate and grow the tube overnight at 30°C, shaking at 250 rpm.
9. Prepare a 15% glycerol stock of *SW106-bmon14272* from the remaining culture and store at –80°C.

D. Prepare Lambda-Red Electrocompetent Cells

1. Chill 20 mL deionized water in a 50 mL conical tube on ice.
2. Add 10 mL of LB media with 10 µL of 50 mg/mL kanamycin to a 50 mL baffled Erlenmyer flask.
3. Inoculate the flask with 250 µL of the remaining *SW106-bmon14272* culture.
4. Grow the culture at 30°C, shaking at 250 rpm to OD_{600} = 0.4–0.6.
5. Shake the culture in a 42°C water bath for 15 minutes.
6. Place the culture on ice until chilled and transfer to a 50 mL conical tube.
7. Spin down the cells in a 4°C centrifuge for 5 minutes at 5,000 × *g*.
8. Pour off the media to a waste container.
9. Resuspend the pellet in 5 mL chilled deionized water by agitating on ice.
10. Add an additional 10 mL chilled deionized water to the sample.
11. Spin the sample in a 4°C centrifuge for 5 minutes at 5,000 × *g*.
12. Pour off the supernatant to a waste container.
13. Resuspend the pellet in 1 mL chilled deionized water by gently pipetting it and moving it to a 1.5 mL tube.
14. Spin the sample in a benchtop centrifuge for 30 seconds at 13,000 × *g*.
15. Remove the supernatant with a pipette and resuspend the sample in 50 µL chilled deionized water by gently pipetting.
16. Place the final sample of competent cells on ice until ready to transform.

E. Inserting CAT-SacB Cassette

1. Add 100 ng of CAT-SacB insertion or knockout cassette to the fresh electrocompetent cells.
2. Carefully move the mixture to the gap in the electroporation cuvette, avoiding bubbles.
3. Transform cells by electroporation of 1.75 kV and immediately flush the electroporation cuvette with 1 mL LB.

4. Remove the transformants from the cuvette by pipetting and place them in a 15 mL Falcon tube.
5. Grow the culture at 30°C, shaking at 250 rpm for 4 hours.
6. Plate 100 µL of the culture on LB agar plates with 50 µg/mL kanamycin and 20 µg/mL chloramphenicol.
7. Grow the plates overnight at 30°C. Colonies grow slowly at this step and may require an additional day of incubation before being able to screen.
8. Pick colonies into 15 mL Falcon tubes with 4 mL LB, 4 µL of 50mg/mL chloramphenicol, and 4 µL of 20 mg/mL chloramphenicol.
9. Grow the cultures overnight at 30°C, shaking at 250 rpm.
10. Add 10 µL of the overnight culture to a 0.2 mL PCR tube and incubate it in the thermocycler at 98°C for 5 minutes.
11. Spin down the cell debris.
12. To a 0.2 mL PCR tube, add the following:
    - 1 µL crude lysate supernatant
    - 0.2 µM forward-confirmation oligo
    - 0.2 µM reverse-confirmation oligo
    - 10 µL 2× Phusion® High-Fidelity PCR Master Mix
    - Deionized water to a final volume of 20 µL
13. Place the tube in the thermocycler and amplify DNA using the following conditions with the Tm calculated from the oligonucleotide pair: 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.
14. Set up electrophoresis equipment and fill it with 1× TAE. Insert a 0.8% agarose gel into the dock.
15. 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.
16. Electrophorese the samples for 1 hour at 100 V.
17. Image the gel with a UV light and verify the insert size, including CAT-SacB, homology arms, and primer flanks.
18. If the cassette appears to have been inserted correctly, make a 15% glycerol stock for the chosen culture.

F. Remove CAT-SacB Markers

1. Prepare lambda-Red electrocompetent cells with the *SW106-bmon14272 -CAT-SacB* strain as in Procedure C, including 42°C induction.
2. Add 100 ng of the removal cassette from the SOP titled “Engineering Cassettes for Scarless Genomic Manipulation” or the 80-mer ssDNA removal oligo.
3. Carefully move the mixture to the gap in the electroporation cuvette, avoiding bubbles.
4. Transform the cells by electroporation of 1.75 kV and immediately flush the electroporation cuvette with 1 mL LB.
5. Remove the transformants from the cuvette by pipetting and place them in a 15 mL Falcon tube with 9 mL of 1× M9 media.
6. Grow at 30°C, shaking at 250 rpm for 4 hours.
7. Pellet 1 mL of culture and put in a benchtop centrifuge for 30 seconds at 13,000 × *g*.
8. Remove the media and wash it with 1 mL of 1× M9 salts. Repeat spin.
9. Resuspend the cells in 100 µL of 1× M9 salts and plate on LB no salt, 6% sucrose agar plates with 100 µg/mL ampicillin.
10. Grow the plates overnight at 30°C, shaking at 250 rpm.
11. Pick colonies into 15 mL Falcon tubes with 4 mL LB and 100 µg/mL ampicillin.
12. Grow the cultures overnight at 30°C, shaking at 250 rpm.
13. Add 10 µL of the overnight culture to a 0.2 mL PCR tube and incubate it in the thermocycler at 98°C for 5 minutes.
14. Spin down the cell debris.
15. To a 0.2 mL PCR tube, add the following:
    - 1 µL crude lysate
    - 0.2 µM forward-confirmation oligo
    - 0.2 µM reverse-confirmation oligo

- 10 µL 2× Phusion® High-Fidelity PCR Master Mix
- Deionized water to a final volume of 20 µL

16. Place the tube in the thermocycler and amplify DNA using the following conditions with the Tm calculated from the oligonucleotide pair: 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.
17. Set up electrophoresis equipment and fill with 1× TAE. Insert 0.8% agarose gel into the dock.
18. 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.
19. Electrophorese samples for 1 hour at 100 V.
20. Image the gel with UV light and verify the insert size, including the size of the altered region and primer flanks.
21. If the cassette appears to have been inserted correctly, make a 15% glycerol stock for the chosen culture.
22. Purify the remaining PCR product and submit it for Sanger sequencing.

G. Extract Bacmid DNA

1. Pellet 2 mL of the remaining *SW106-bmon14272-CAT-SacB* culture in a 2 mL tube at 13,000 × *g*.
2. Aspirate off the supernatant and resuspend the pellet in 250 µL of Plasmid Prep Buffer A by pipetting.
3. Add 250 µL Plasmid Prep Buffer B and mix by inverting 6 times.
4. Add 250 µL Plasmid Prep Buffer C and mix by inverting 6 times.
5. Centrifuge the sample at 13,000 × *g* for 10 minutes and transfer 700 µL of supernatant to a fresh 1.5 mL tube.
6. Centrifuge the supernatant at 13,000 × *g* for 10 minutes and transfer 600 µL of supernatant to a fresh 2 mL tube.
7. Add 1.2 mL of room temperature 100% ethanol to the supernatant and mix by inversion 6 times.
8. Centrifuge the sample at 13,000 × *g* for 20 minutes to pellet DNAs.
9. Carefully remove the supernatant, avoiding the pellet, and dispose of the liquid in an appropriate alcohol waste container.
10. Centrifuge the sample at 13,000 × g for 30 seconds.
11. Carefully remove any remaining supernatant, avoiding the pellet, and dispose of the liquid in an appropriate alcohol waste container.
12. Add 100 µL of Plasmid Resuspension Buffer to the pellet and shake for 1 hour at 37°C.
13. Store the mixture at –20°C until ready for transformation.

H. Prepare Final BEVS Strain

1. Prepare Electrocompetent DE96 or similar Dh10Bac-derivative strain, including helper plasmid using the protocol above in Procedure B, and add 50 µg/mL kanamycin and 12 µg/mL tetracycline to the growth media.
2. Add 1 µL of extracted mutant bacmid DNA to the DE96 electrocompetent cells on ice.
3. Carefully move the mixture to the gap in an electroporation cuvette, avoiding bubbles.
4. Electroporate the mixture with 1.75 kV and immediately flush it with 1 mL LB.
5. Remove the transformants by pipetting into a fresh 15 mL Falcon tube and incubate the tube at 30°C with shaking for 1 hour.
6. Plate 100 µL of the culture on LB agar plates with 50 µg/mL of kanamycin and 12 µg/mL of tetracycline and incubate it overnight at 30°C.
7. Add 4 mL LB, 4 µL of 50 mg/mL kanamycin, and 4 µL 12 mg/mL tetracycline to a 15 mL Falcon tube.
8. Inoculate the media with a single colony from the *DE96-mutant bacmid* transformant plate.
9. Grow the culture at 30°C overnight, shaking at 250 rpm.
10. Prepare a 15% glycerol stock for inventory of the new bacmid strain.
11. The strain construction is now complete, and you can prepare chemically competent cells for use with Bac-2-Bac protocols using pFastBac or similar expression plasmids to introduce your gene(s) of interest.

References

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