Protein Science - Generation of Recombinant Bacmid DNAs

Purpose

To generate bacmid DNAs containing the gene you wish to overexpress in insect cells.

Scope

This protocol details the construction of bacmid DNAs where a baculovirus-expression-system-style expression clone is transformed into an *E. coli* strain containing a baculovirus shuttle vector and transposases on a helper plasmid. Once the transposition is confirmed, bacmid DNA is extracted to be used for propagating recombinant baculovirus.

Definitions

PCR: Polymerase Chain Reaction

Materials and Equipment

- Oligonucleotides, 5 µM (details below)
- Phusion High-Fidelity Master Mix (New England Biolabs, cat. #M0531S)
- Baculoviral expression clone (generated in SOP Combinatorial Assembly of Clone Libraries Using Site-Specific Recombination) or similar pFastBac-style plasmid containing your gene of interest
- Chemically competent cells of an *E. coli* bacmid strain, such as DH10Bac, DE95, etc.
- Lysogeny broth (LB) media (Fisher Scientific, cat. #BP1427-500)
- LB agar plates with 50 µg/mL kanamycin, 10 µg/mL tetracycline, 7 µg/mL gentamycin, 40 µg/mL IPTG, and 100 µg/mL Bluo-gal (Teknova, SKU #L1919)
- 1.5 mL tubes (USA Scientific, item #1615-5500)
- 2.0 mL tubes (USA Scientific, item #1620-2700)
- Deionized water
- 10× TE (Fisher BioReagents, cat. #BP2477-1) (diluted to 1× with water, filter-sterilized)
- Ethanol
- 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.
  - If precipitation has occurred, redissolve before use by heating at 37°C.
  - Make a fresh batch 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, cat. #12091021])
  - Store at room temperature for up to 6 months.
- 0.2 mL PCR tubes (USA Scientific, item #1402-8100)
- Falcon 2059 culture tubes (Fisher Scientific, item #14-959-11B)
- 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, SKU #X4126], 3% tartrazine [Sigma, SKU #T0388], 50% glycerol) (diluted to 2× with deionized water)
- 1 kb Plus DNA Ladder (New England Biolabs, cat. #N3200L) (100 µL DNA ladder, 20 µL 10× gel loading dye, 350 µL 1× TE, 5 µL 0.5M EDTA [pH 8.0])
- Agarose gel 0.8% with ethidium bromide (Embi Tec, cat. #GE-3701)
- 10× TAE (Embi Tec, cat. #EC-1016) (diluted to 1× with water)
- Glycerol (Sigma, SKU #G5516) (diluted to 60% with water, filter-sterilized)
- 37°C incubator with shaking platform
- Benchtop centrifuge
- Thermocycler

- Electrophoresis equipment
- Gel imager with ultraviolet 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. Oligonucleotide Design

To confirm the transposition event, Thermo Fisher recommends using pUC/M13 forward and reverse primers to amplify across the entire insert from a pFastBac plasmid. We prefer to use four primers (Baccheck-R, Baccheck-F, BaccheckBtB-L, BaccheckBtB-R) to multiplex the two transposition junctions. Oligonucleotides can be synthesized at whatever concentration you prefer and then diluted to 5 µM with 1× TE. For the multiplex junction PCR, the oligonucleotides can be pooled at equal volumes.

Primer Name	Primer Sequence 5′-3′
pUC/M13 Forward	CCCAGTCACGACGTTGTAAAACG
pUC/M13 Reverse	AGCGGATAACAATTTCACACAGG
Baccheck-R	GTGCTGCAAGGCGATTAAGT
Baccheck-F	TGTGGAATTGTGAGCGGATA
Baccheck BtB-L	ATCAGCCGGACTCCGATTA
Baccheck BtB-R	CCCACACCTCCCCCTGAACCTG

B. Transformation of Baculoviral Expression Clone into Bacmid Strain

1. Thaw on ice 50 µL of DE95, DH10Bac, or a similar bacmid strain.
2. Add 1 µL (100–200 ng) of the baculoviral expression clone to the thawed cells.
3. Incubate the mixture on ice for 15 minutes.
4. Heat-shock the cells at 42°C for 45 seconds and immediately place them back on ice for 2 minutes.
5. Add 450 µL of LB and place all transformants into a Falcon 2059 tube.
6. Shake the tube for 4 hours at 37°C.
7. Add 48 µL LB and 2 µL of the transformants to an LB agar plate with 50 µg/mL kanamycin, 10 µg/mL tetracycline, 7 µg/mL gentamycin, 40 µg/mL IPTG, 100 µg/mL Bluo-gal.
8. Grow the cells overnight at 37°C.
9. Pick two or three white colonies to Falcon 2059 tubes containing 3 mL LB media with 50 µg/mL kanamycin and 7 µg/mL gentamycin.
10. Grow the colonies overnight at 37°C, 250 rpm.

C. Preparation of Bacmid DNAs

1. Pellet 2 mL of overnight culture in a benchtop centrifuge at max speed for 1 minute.
2. Aspirate the supernatant.
3. Add 250 µL of Plasmid Prep Buffer A to the tube and resuspend the pellet by vortexing.
4. Add 250 µL of Plasmid Prep Buffer B and resuspend the pellet by inverting the tube 6–10 times. The mixture should become viscous.
5. Add 250 µL of Plasmid Prep Buffer C and resuspend the pellet by inverting the tube 6–10 times. The mixture should have visible white flocculation of cell debris.
6. Spin the tube at max speed in a benchtop centrifuge for 10 minutes.
7. Remove 700 µL of supernatant to a fresh 1.5 mL tube.
8. Spin the tube at max speed in a benchtop centrifuge for 10 minutes.
9. Remove 600 µL of supernatant to a fresh 2 mL tube.
10. Add 1.2 mL ethanol to the tube and invert the tube 6–10 times to mix.
11. Spin the tube at max speed in a benchtop centrifuge for 20 minutes.
12. Pour off the supernatant.
13. Spin the tube at max speed in a benchtop centrifuge for 1 minute.
14. Aspirate the remaining alcohol mixture, avoiding the white pellet.
15. Allow the tube to dry for 10 minutes at room temperature.
16. Add 100 µL of Bacmid Resuspension Buffer and place the culture in a 37°C shaker for 1 hour.

D. Screening for Correct Transposition

1. Add the following to a 0.2 mL PCR tube:

   a. 1 µL of bacmid DNA prep
   b. 1 µL of 5 µM M13 forward and reverse primers, or pooled junction oligo mix
   c. 8 µL deionized water
   d. 10 µL Phusion High-Fidelity Master Mix
2. Add the tube to a thermocycler with the following conditions: initial denaturation at 98°C for 45 seconds, followed by 30 cycles (each 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds—or 30 seconds per kb if amplifying a full insert with M13 primers), followed by a 10-minute final elongation at 72°C and cooling to 4°C.
3. Set up electrophoresis equipment and fill it with 1× TAE. Insert 0.8% agarose gel into the dock.
4. 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 the 1 kb Plus DNA Ladder with loading dye in a neighboring well.
5. Electrophorese for 1 hour at 100V.
6. Image the gel with ultraviolet light. The expected amplicons in the multiplex reaction will be 570 bp/840 bp. The expected amplicon in the M13 reaction will be the entire expression clone insert (between Tn7 left and right arms).
7. If the amplicons are correct, make a 15% glycerol stock of the correct construct and store it at –80°C.
8. The prepared DNA contains *E. coli* genomic DNA, residual expression and helper plasmids, and the bacmid DNA, so concentration for the bacmid DNA cannot be accurately determined.
9. Reserve this DNA at –20°C until you are ready to prepare the baculovirus.

References

1. Luckow VA, Lee SC, Barry GF, and Olins PO (1993). Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. *J Virol.* 67:4566–4579. doi: 10.1128/JVI.67.8.4566-4579.1993.
2. Gillette W, Frank P, Perkins S, Drew M, Grose C, Esposito D (2019). Production of Farnesylated and Methylated Proteins in an Engineered Insect Cell System. *Methods Mol Biol.* 2009:259-277. doi:10.1007/978-1-4939-9532-5_20.