Baculovirus protein expression methods

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pFastBac cloning

1. Design blunt-end primers (without 5' phosphates) for the gene of interest
2. Perform a PCR reaction with the following conditions:

Reagent	Volume	Concentration
Sense primer	1μl	1μg/μl
Antisense primer	1μl	1μg/μl
Pfu Pol buffer	5μl	10x
DNA Template	50ng **Total DNA**	-
Sterile Filtered UV/UF H_2O	Bring volume to 44.5	-
Pfu Turbo	0.5μl	5000 U/ml

*Use thermocyling conditions appropriate for your insert

***Heat to 80°C and add 5μl DNTP**

3. Mix the following from PCR products and Invitrogen kit - pFastBac TOPO:
    1:1 ratio of Vector:Insert (Vector Conc. 10ng/μl)
    1uL Salt solution
    1uL pFastBac TOPO vector
    Total 6μl
4. Mix reagents gently; incubate at room temp for 5 minutes
5. Place reaction on ice
6. ThawOneShot Mach1 T1 on ice
7. Mix 2uL pFastBac reaction product gently with Mach 1 cells
8. Incubate on ice for 30 min
9. Heat shock at 42°C for 30 sec
10. Immediately incubate cells on ice for 2 min
11. Add 250 μL SOC medium and shake for 1 hour at 37°C
12. Plate on LB + Amp plates overnight.
13. Screen using colony PCR – use insert-specific sense and SV40 antisense primers
14. Isolate colonies and amplify each separately in 30mL LB + ampicillin (100 ug/mL) overnight
15. Purify vector DNA from pellet via QIAGEN Plasmid Prep kit
16. Prepare sequencing reactions:
    Submit one forward AND one reverse reaction for EACH colony selected
    Each reaction tube should have the following:
        10 μL of Forward OR Reverse primer (concentration of 0.8 picomol/microliter)
        10 μL concentrated DNA product (~100ng/μL)
    Forward Primer: Polyhedrin promoter primer (MC)
    Reverse Primer: SV40 polyA reverse primer (MC)
        *Both primers are stored with the TOPO kit in the -20C small bench freezer
17. Analysis of sequencing reactions:
    A. Ensure that the insert is present and in-frame
    B. Ensure that the insert is in the correct orientation

Bacmid generation

Prepare plates before transformation:
-Triple antibiotic selection is required throughout this process
Prepare antibiotic stocks:
Kanamycin: 50 μg/mL = 0.25 g in 5 mL for 1000X stock (in H2O)
Gentamycin: 7 μg/mL = 0.035 g in 5mL for 1000X stock (in H2O)
Tetracycline: 10 μg/mL = 0.05 g in 5mL for 1000X stock (in Ethanol)
Autoclave LB agar and add antibiotics to 1x

-IPTG and Xgal are used for blue white colony selection
-Use Kan/Gent/Ter plate
IPTG: 0.1M stock solution
-spread 40μL per plate after plates have cooled
Xgal: 20 mg/mL stock solution in DMSO
-spread 40μL per plate after plates have cooled
Let plates dry in incubator

1. Thaw 1 half of a vial (50μL) of DH10Bac cells on ice for 15 min
2. Add 50 ng pFastBac vector DNA (ideally 1μL of 50ng/μL) to cells
3. Mix gently and incubate on ice for 30 min
4. Heat shock at 42° C for 45 sec
5. Immediately incubate on ice for 2 min
6. Add 450 μL SOC media, shake at 37° C for 6 hours
7. Plate Cells (Kanamycin, Gentamycin, Tetracycline, IPTG, XGAL)
    No dilution (100μL per plate)
    1:10 dilution
    1:100
8. Incubate plates inverted at 37°C for at least 24-36 hours (Picking colonies too early will give false positives)
9. Select 10 colonies for PCR
10. Perform colony PCR on selected white colonies to ensure recombination occurred

Reagent	Volume	Concentration
Insert Specific Sense primer	1μl	1μg/μl
M13 Antisense primer	1μl	1μg/μl
ThermoPol buffer	5μl	10x
DNA Template	50ng **Total DNA**	-
Sterile Filtered UV/UF H_2O	Bring volume to 44.5	-
Taq Polymerase	0.5μl	5000 U/ml

*Use thermocyling conditions appropriate for your bacmid + insert (a long extension time, ~3:30min, will be necessary)
***Heat to 80°C and add 5μl DNTP**

11. Analyze product via agarose gel electrophoresis (size equals 2440 bp + insert)

Amplification and Purification of Bacmid

1. Inoculate small seed culture (5-10mL) LB + triple antibiotic selection (same concentration as plates from DH10Bac transformation), grow for ~8 hours
2. Transfer seed culture to 500mL LB culture supplemented with 10 g NaCl (to increase bacmid yield) and triple antibiotic selection
3. Grow overnight
4. Harvest pellet (centrifuge at 6000 rpm for 30 min)
5. Purify bacmid with QIAGEN Large Construct Kit
6. Elute in TE buffer pH 8.0
7. Measure bacmid concentration via UV-Vis
8. Store bacmid at 4°C for up to 2 weeks – Store at -20°C for long term use avoid repeated freeze-thaw cycles
9. Run bacmid on a 1% agarose gel to assess its integrity, multiple small bp bands indicate degradation of bacmid

Transfecting insect cells (P1)

1. Ensure that Sf9 cells are in log phase (This can be done by splitting them back 1-2 days before transfection, cell should be at ~$5x10^5$ cells/mL, 95% viable)
2. Add a total of $8x10^5$ cells to 6 well plate
3. Add 2ml of **unsupplemented** Grace's insect media (**No FBS or antibiotics**)
4. Incubate in hood for 15 minutes to allow cells to attach
5. During incubation add 8µl cellfectin reagent and 2.5µg bacmid to 200µl **unsupplemented** Grace's media, incubate 15-30 minutes, mix by agitation
6. Add transfection mixture (cellfectin and bacmid DNA) dropwise to 6 well plate
7. Incubate at 27°C for 6-8 hours
8. Remove media and add 2ml supplemented Grace's media (10% FBS, 1% Penn/Strep)
9. Incubate at 27°C for 72 hours then check for signs of viral transfection:

Signs of Infection	Phenotype	Description
Early (first 24 hours)	Increased cell diameter	A 25–50% increase in cell diameter may be seen.
	Increased size of cell nuclei	Nuclei may appear to "fill" the cells.
Late (24–72 hours)	Cessation of cell growth	Cells appear to stop growing when compared to a cell-only control.
	Granular appearance	Signs of viral budding; vesicular appearance to cells.
	Detachment	Cells release from the plate or flask.
Very Late (>72 hours)	Cell lysis	Cells appear lysed, and show signs of clearing in the monolayer.

10. Transfection may take up to 5 days
11. Remove media and centrifuge 10 min at 4000rpm
12. Keep supernatant – store at 4°C for short term use or -80°C for long term storage **Keep at 4°C for routine use, Protect from light**

Baculovirus Amplification

Two methods for amplification:

Method A:

1. Prepare a 50ml culture with a cell density of $2x10^6$ cells/ml (cells should be in log phase) in a shaker flask
2. Add 1ml of P1 stock to shaker flask
3. Incubate in shaker at 27°C for 48-72hrs
4. Check daily for cell growth and death (Infected cell will have decreased growth rates and high cell death 50-70%), use hemocytometer to determine cell concentration each day
5. Remove media and centrifuge 10 min at 4000rpm
6. Store at 4°C for short term and -80°C for long term storage

Advantages – Faster amplification, less opportunity for mutations to arise in baculovirus
Disadvantage – Less robust, produces lower titer

Method B:

1. Add $2x10^6$ cells to a 6 well plate with 2mL Supplemented Grace's Media
2. Incubate 30 min to allow adherence
3. Add 1mL P1 stock
4. Monitor cells for signs of viral infection
5. Harvest media ~48hrs post-infection
6. Store at 4°C until use
7. Prepare a 50ml culture with a cell density of $2x10^6$ cells/ml (cells should be in log phase) in a shaker flask
8. Add 1ml of P2 stock to shaker flask
9. Incubate in shaker at 27°C for 48-72hrs
10. Check daily for cell growth and death (Infected cell will have decreased growth rates and high cell death 50-70%), use hemocytometer to determine cell concentration each day
11. Remove media and centrifuge 10 min at 4000rpm
12. Store at 4°C for short term and -80°C for long term storage

Advantages – Robust system for viral stock generation produces high titer stock
Disadvantages – Slower amplification, increased opportunity for mutations in baculovirus

*Use a viral plaque assay to determine titer

<u>Baculovirus PCR</u>

*This step is optional but verifies that the inserted gene is still included in the bacmid
Protocol adapted from Journal of Virological Methods, Volume 148, Issues 1–2, March 2008, Pages 286–290

1. Take 100µl of the P2 baculovirus stock
2. Add one volume (100µl) lysis buffer (10 mMTris–HCl pH 7.6; 10 mM EDTA, 0.25% SDS)
3. Add one volume of chloroform (200µl)
4. Invert for 4 minutes
5. Centrifuge 5 min at 14000 x g
6. Recover aqueous phase ~100µl
7. Add 10µl sodium acetate pH 5.2
8. Add 450µl 90% ethanol
9. Centrifuge 15 min at 14000 x g
10. Discard supernatant

11. Dry pellet for 5 min at 60°C (ensure ethanol is completely evaporated or DNA may not go into solution)
12. Resuspend in 50µl Tris buffer (10mmM pH 8.5)
13. Run PCR using method from bacmid generation

MOI optimization

1. Prepare 50ml flask of Sf9 cells at $1.5x10^6$ cells/ml
2. Add Baculovirus at an MOI of 1, 5, and 10 using the following equation:

$$\text{Inoculum required (ml)} = \frac{\text{MOI (pfu/cell)} \times \text{number of cells}}{\text{titer of viral stock (pfu/ml)}}$$

3. Monitor cell health daily throughout the process
4. Take 1ml aliquots 2, 3, 4, and 5 days post infection
5. Centrifuge aliquots
6. Resuspend pellet in 30µl lysis buffer (200mM NaCl, 50mM Tris, pH 8)
7. Boil cells for **at least 10min** with SDS PAGE loading dye
8. Run a western blot

Alternatively, see "Baculovirus and Insect Cell Expression Protocols" 2007. CH 10, Small-Scale Protein Production with the Baculovirus Expression Vector System. for a 24 well expression optimization protocol *Method requires validation but appears more useful

Protein expression and purification

1. Infect Sf9 culture ($1.5x10^6$cells/ml) with optimized MOI
2. Incubate for optimized time
3. Centrifuge cell suspension for 10min at 4000rpm
4. Purify protein from cell pellet

Sf9 Culture Maintenance

Media: Sf-900 II SFM + 10% FBS + 1% Penicillin/Streptomycin – Store at 4°C, protected from light

Maintain at 27°C, 125 rpm

Doubling time ~26 Hrs

For suspension cultures – Passage cells at ~$3x10^6$ cells/mL
Seed cultures at ~$4x10^5$ cells/mL
*Cells should be passaged at least every 4 days