Chapter 5

Screening and Production of Recombinant Human Proteins: Protein Production in Insect Cells

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Abstract

This chapter describes the step-by-step methods employed by the Structural Genomics Consortium (SGC) for screening and producing proteins in the baculovirus expression vector system (BEVS). This eukaryotic expression system was selected and a screening process established in 2007 as a measure to tackle the more challenging kinase, RNA–DNA processing, and integral membrane protein families on our target list. Here, we discuss our platform for identifying soluble proteins from 3 mL of insect cell culture and describe the procedures involved in producing protein from liter-scale cultures.

Key words Insect cells, Baculovirus, BEVS, Expression, Recombinant, Protein, Purification, IMAC, SEC chromatography, Gel filtration

1 Introduction

Availability of a pure protein is essential for obtaining information on protein structure and function. Heterologous protein production in *E. coli* has remained the preferred system for many research laboratories as it is low-cost, fast, and easy to handle. However, there is no guarantee that *E. coli* cells will produce eukaryotic proteins in a soluble and biologically active form because of a number of limitations such as codon bias, lack of posttranslational modifications (PTMs), or disulfide bond formation. Exploring other protein expression hosts such as mammalian cells, yeast, and insect cells is often required if *E. coli* fails to produce soluble protein after attempting different strains, solubility enhancing tags, and so on. Among the alternatives available, the baculovirus expression vector system (BEVS) is increasingly becoming popular for expression of recombinant proteins as it is nonpathogenic to humans [1], capable of producing high levels of soluble proteins with PTMs similar to those observed in mammalian cells and easily scalable in

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suspension culture [2]. This system is also proving popular for the production of large protein complexes, production of virus-like particles, gene delivery, viral vector vaccines, expression of proteins in mammalian cells, and display of proteins and peptides on the baculovirus envelope [3]. Baculoviruses are double-stranded DNA viruses [4] most of which infect insects of the order Lepidoptera [5]. The most widely used baculovirus used as a BEVS is *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Two major genes that express in the very late phase of baculovirus infection of insects are p10 and polyhedrin which are strong expressers but dispensable for viral replication. This discovery has allowed for exploitation of the p10 and polyhedrin promoters to be used for driving recombinant protein expression in BEVS; the polyhedrin promoter in particular has been described as a workhorse promoter of BEVS [6]. The most common insect cell lines utilized as hosts of BEVS are Sf9 and Sf21 derived from pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda* [7] and High Five cells (BTI-Tn-5B1–4) derived from ovarian cells of the cabbage looper, *Trichoplusia ni* [8].

Since the first use of baculoviruses for protein expression in 1983 [9], the system has gone through numerous technological advances that have allowed it to be widely accessible. Various baculovirus expression systems are commercially available to produce baculoviruses, most notably Bac-to-Bac® (Invitrogen), flashBAC (Oxford Expression Technologies), BaculoDirect™ (Invitrogen), BacVector®-3000 (Novagen), BacPAK (Clontech), and Bac-n-Blue™ (Invitrogen). About 12 years ago, it became evident in our laboratory that the bacterial expression system was unable to cope with more challenging proteins on our target list such as many protein kinases, RNA–DNA processing proteins, and integral membrane proteins (IMPs). To address this issue, we established an efficient process based on the Bac-to-Bac® system [10] for screening multiple versions of each protein in insect cells to identify those that were amenable to purification and crystallization. The 96-well cloning procedure is described in detail in Chapter 3. In this chapter we continue the methodologies for expression screening and scaling up expression of proteins in suspension culture. To describe our series of standardized protocols for protein production in insect cells, this chapter is broadly divided into the following stages: (a) transposition, bacmid production and PCR screen; (b) growth and maintenance of insect cell lines in adherent and suspension culture; (c) transfection into Sf9 cells, baculovirus generation, and small-scale test expression/purification; and (d) large-scale protein expression and purification. The screening process has been miniaturized to 24-well format. The steps involved in the pipeline from cloning to large-scale expression are outlined in Fig. 1.

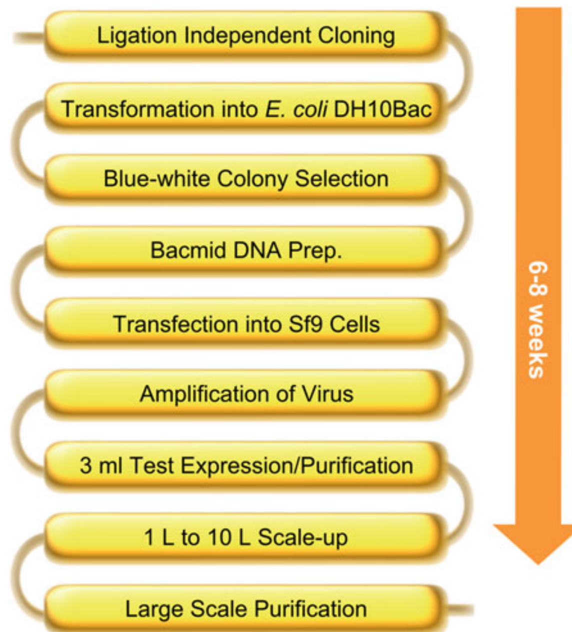

Fig. 1 Overview of the Baculovirus expression process. The process takes ~6–8 weeks from LIC to scale-up

2 Materials

Unless otherwise stated, all solutions are prepared using ultrapure water (prepared by purifying deionized water to reach a resistivity of 18 MΩ cm at 25 °C) and analytical grade reagents.

2.1 Transposition and Bacmid Preparation

1. *E. coli* DH10Bac (Invitrogen) or DH10EMBacY (Geneva Biotech) chemically competent bacterial cells are prepared in house as described [11] (*see* **Note 1**).
2. Primers: Primers are supplied by Eurofins and are HPSF purified at 0.01 or 0.05 μmol scale. Primer stocks are either supplied at or diluted (in 10 mM Tris–HCl buffer, pH 8.0) to 100 μM and stored at -20 °C.
3. MyTaq™ Red DNA Polymerase (5 unit/μL, Bioline).
4. Molecular biology grade water.
5. 10 mM dNTP solution: 2.5 mM dATP, 2.5 mM dTTP, 2.5 mM dGTP, and 2.5 mM dCTP (prepare from 100 mM dNTP set) diluted in molecular biology grade water and stored at −20 °C.
6. 50× TAE buffer (1 L): Dissolve 242 g of Tris base, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA, pH 8.0 in water and adjust pH to 8.5. Filter through a 0.2 μm membrane filter and use as a 1× solution.

7. 96-Well 1.5% TAE-agarose gels: Dissolve 3 g of agarose powder in 200 mL of 1× TAE buffer using a microwave. Once cooled to hand-hot, add 8 μL of SYBR-safe DNA gel stain (Invitrogen), mix by swirling and cast in a Sub-cell Model 96 (Bio-Rad or similar) gel cast.
8. DNA ladder: 1 kb Plus DNA Ladder (Invitrogen) prepared in 1× BlueJuice™ (Invitrogen) diluted in molecular biology grade water.
9. TE Buffer: Prepare a solution of 10 mM Tris–HCl and 1 mM EDTA, pH 8.0, filter through a 0.20 μm syringe filter and store at room temperature (RT).
10. 60% (v/v) glycerol: Autoclave to sterilize.
11. 70% (v/v) ethanol.
12. 50 mg/mL kanamycin: Prepare in water, filter through a 0.20 μm syringe filter and store at −20 °C.
13. 10 mg/mL tetracycline: Prepare in ethanol and store −20 °C.
14. 7 mg/mL gentamycin: Prepare in water, filter through a 0.20 μm syringe filter, and store at −20 °C.
15. 100 mg/mL Blue-gal (Glycosynth): Prepare in dimethyl sulfoxide (DMSO) and store −20 °C.
16. 40 mg/mL IPTG: Prepare in water, filter through a 0.20 μm syringe filter, and store at −20 °C.
17. LB agar: Dissolve 22.5 g of premixed LB broth and 13.5 g of agar in 800 mL of ultrapure water. Adjust volume to 900 mL and autoclave on the same day.
18. Recombinant bacmid selection plates: Melt LB agar slowly in a microwave and add 5% (w/v) sucrose. Once cooled to hand-hot, add the appropriate antibiotic and swirl vigorously to mix. Pour 10 mL of the molten agar into each 50 mm petri dish and once set, upturn and leave open to dry. These can be prepared ahead of time and stored for up to a month at 4 °C, sealed in a plastic bag to prevent overdrying.
19. 2× LB: Dissolve 45 g of premixed LB broth in 800 mL of water. Adjust volume to 900 mL and autoclave on the same day.
20. Virkon.
21. Montage Plasmid Miniprep$_{HTS}$ 96 Kit (Millipore, *see* **Note 6**).
22. 50 mm petri dishes.
23. 96-well PCR plates.
24. 96-well microtiter plates that can hold up to 200 μL of sample.
25. 96-deep-well blocks.
26. Adhesive tape pads.

27. 96-well filter plates, 25 μm.
28. Adhesive PCR seals.
29. AirOtop porous seals (Thomson or VWR).
30. Silicone 96-Square-Well AxyMat (Axygen).
31. Disposable sterile spreaders or 2 mm autoclaved glass balls (VWR).
32. Disposable sterile inoculation loops (1 μL).
33. Reagent reservoirs for multichannel pipetting.
34. Minisart syringe filters, 0.20 μm.
35. Supor® PES Membrane Disc Filters, 0.2 μm and unit (Pall).
36. Multichannel pipettes and repeat pipettors are used to dispense reagents into a 96-well format.
37. 96-well PCR thermocycler with heated lid.
38. 96-well gel cast and tank (Subcell Model 96 Bio-Rad or similar).
39. All gels are imaged on a Gel Doc™ XR+ (Bio-Rad).
40. A UV spectrophotometer for measuring DNA and protein concentration (e.g., The NanoDrop™ spectrophotometer allows for measurements from as low as 1.5 μL volumes).
41. Scanlaf Mars recirculating class II biological safety cabinet (BSC).
42. Micro-Express Glas-Col shaker (Glas-Col, Indiana, USA) or alternative that ranges in temperature from 18 °C to 37 °C and shakes up to 800 rpm.
43. 96-well block mixer (Eppendorf MixMate or similar).
44. Water bath set at 42 °C.
45. Incubator set at 37 °C.
46. Centrifuge suitable for 96-deep-well blocks (3,000 × *g*).

2.2 Transfection and Cell Growth

The following reagents, consumables, and equipment are required in addition to those listed above:

1. Cell lines: Sf9 insect cells, SFM adapted (Invitrogen); High Five cells, SFM adapted (Invitrogen).
2. Media: Sf-900™ II SFM (1×) (Invitrogen).
3. Reagents: fetal bovine serum (FBS), insect cell culture tested (Invitrogen); Insect GeneJuice® (Merck), Pen/Strep (use at 50 units penicillin and 50 μg streptomycin per mL of medium); 0.4% Trypan Blue Stain.
4. DMSO, Molecular Biology grade (DNase/RNase free).
5. Cryovials.

6. 24-well tissue culture plates.
7. 24-well blocks (Microplate Devices Uniplate® or similar).
8. 250, 500, and 1,000 mL flasks with vented cap (Corning).
9. Stripette pipettes.
10. Inverted light microscope (Axiovert 25, CarlZeiss).
11. Hemocytometer, improved Neubauer (VWR International).
12. Static incubator set at 37 °C.
13. Multitron shaker-incubators with cooling capacity (Infors HT).

2.3 Virus Amplification and Test Expression

All reagents, consumables, and equipment listed above.

2.4 Test Purification

The following reagents, consumables, and equipment are required in addition to those listed above:

1. Benzonase (Novagen, HC, 250 units/μL).
2. Protease Inhibitor Cocktail Set III (Calbiochem).
3. 0.5 M Tris(2-carboxyethyl)phosphine (TCEP): Prepare in water, filter through a 0.20 μm syringe filter, and store at −20 °C.
4. 1 M dithiothreitol (DTT): Prepare in water, filter through a 0.20 μm syringe filter, and store as 1 mL aliquots at -20 °C.
5. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).
6. InstantBlue™ (Expedeon Protein Solutions).
7. 20× NuPAGE™ MES SDS Running Buffer (Invitrogen).
8. PBS: Dissolve 5 tablets of PBS in 1 L of water, filter through a 0.2 μm membrane filter, and store at 4 °C.
9. 1 M HEPES, pH 7.5: Prepare in water, filter through a 0.2 μm membrane filter, and store at RT.
10. 5 M NaCl: Prepare in water, filter through a 0.2 μm membrane filter, and store at RT.
11. 3 M imidazole, pH 8.0: Prepare in water, filter through a 0.2 μm membrane filter, and store at RT.
12. 50% (v/v) glycerol: Autoclave and store at RT.
13. Lysis buffer (1 L): 50 mM HEPES, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, and 10 mM imidazole prepared in advance, filtered through a 0.2 μm membrane filter and stored at 4 °C. On the day of purification, add 0.2 μL/mL Benzonase, 1 μL/mL protease inhibitor cocktail, and 0.5 mM TCEP.

14. Wash buffer (1 L): 50 mM HEPES, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, and 30 mM imidazole prepared in advance, filtered through a 0.2 μm membrane filter and stored at 4 °C. Add 0.5 mM TCEP on the day of purification.
15. Elution buffer (0.1 L): 50 mM HEPES, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, and 500 mM imidazole prepared in advance, filtered through a 0.2 μm membrane filter and stored at 4 °C. Add 0.5 mM TCEP on the day of purification.
16. Affinity buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, and 10 mM imidazole prepared in advance, filtered through a 0.2 μm membrane filter, and stored at 4 °C.
17. 50% (w/v) Ni-IDA Metal Chelate Resin (Generon) or Ni-NTA-agarose (Qiagen): The IMAC resins are generally supplied in 20% ethanol. To equilibrate, wash the resin twice in water and then three times in Affinity buffer in a 50 mL tube, by inverting to resuspend the resin and centrifuging at 500 × *g* for 1 min. After the final wash, resuspend the resin in Affinity buffer as 50% (w/v) slurry and store at 4 °C when not in use.
18. SB: Prepare a stock of NuPAGE LDS sample buffer (Invitrogen) containing DTT (1:4 dilution of 1 M DTT in NuPAGE LDS sample buffer) and store at −20 °C.
19. 96-Well filter plates.
20. Precast 26-Lane SDS-PAGE gradient gels (4–12% Bis-Tris) (Invitrogen).
21. Protein gel electrophoresis apparatus (Invitrogen).
22. 96-Well thermocycler with heated lid.
23. Vibra-Cell Sonicator with 24-well probe (Sonics®).
24. General purpose benchtop centrifuge (Sorvall Legend RT, Kendro).

2.5 Large-Scale Expression

The following reagents, consumables, and equipment are required in addition to those listed above:

1. Media: Sf-900™ II SFM (1×) (Invitrogen); Insect-XPRESS serum-free and protein-free medium (Lonza).
2. Nonbaffled Erlenmeyer flasks: glass or polycarbonate in various sizes 250 mL, 500 mL, and 1 L and glass flasks of 3 L capacity for large-scale expression.
3. Cell freezing container: Mr. Frosty (Nalgene).
4. Avanti J-20XP or Avanti J-26XP centrifuge or similar (Beckman Coulter) with a JLA 8.1000 rotor for harvesting large volumes of cells.
5. Chemgene.
6. Alconox®.

2.6 Protein Extraction and Large-Scale Purification

The following reagents, consumables, and equipment are required in addition to those listed above.

1. Complete EDTA-free protease inhibitor (Roche).
2. 2× Lysis buffer: 100 mM HEPES buffer, pH 7.5, 1 M NaCl, 20% (v/v) glycerol, and 20 mM imidazole. Filter through a 0.2 μm membrane filter and store at 4 °C. On the day of purification, add Benzonase (0.2 μL/mL of cell lysate), Protease inhibitor cocktail (2 μL/mL of cell lysate) or Complete EDTA-free protease inhibitor cocktail (1 tablet/25 mL of cell lysate), and 1 mM TCEP.
3. Lysis buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, and 10 mM imidazole. Filter through a 0.2 μm membrane filter and store at 4 °C. On the day of purification, add Benzonase (0.1 μL/mL of cell lysate), Protease inhibitor cocktail (1 μL/mL of cell lysate) or Complete EDTA-free protease inhibitor cocktail (1 tablet/50 mL of cell lysate), and 0.5 mM TCEP.
4. Affinity buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, and 10 mM imidazole. Filter through a 0.2 μm membrane filter and store at 4 °C. Add 0.5 mM TCEP on the day of purification.
5. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, and 30 mM imidazole. Filter through a 0.2 μm membrane filter and store at 4 °C. Add 0.5 mM TCEP on the day of purification.
6. Elution buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, and 300 mM imidazole. Filter through a 0.2 μm membrane filter and store at 4 °C. Add 0.5 mM TCEP on the day of purification.
7. Size Exclusion Chromatography buffer (SEC): 20 mM HEPES, pH 7.5, 500 mM NaCl, and 5% glycerol. Filter through a 0.2 μm membrane filter and store at 4 °C. Add 0.5 mM TCEP on the day of purification.
8. Minisart syringe filters, 0.20 μm, 0.45 μm, and 0.80 μm.
9. Amicon Ultra protein concentrators.
10. Sonicator (Sonics Vibra-Cell, VCX 750, Sonics & Materials INC) or basic Z model cell disruptor (Constant Systems Ltd).
11. ÄKTA-Xpress or ÄKTA-Purifier liquid chromatography system.
12. HiTrap 5 mL FF columns for his-tagged protein purification.
13. Ion exchange chromatography columns such as HiTrap 5 mL Q FF and SP FF.

14. HiLoad Superdex columns for preparative size exclusion chromatography such as HiLoad 16/600 Superdex™ S75 pg, S200 pg, or Superose™ 6 Increase 10/300 GL.
15. JA-25.50 rotor for centrifugation of cell lysates.

3 Methods

3.1 Transposition in E. coli DH10Bac or DH10EMBacY

The transposition process is outlined in Fig. 2.

1. Prepare at least 100 petri dishes (50 mm) containing approximately 10 mL of LB agar, supplemented with 50 μg/mL kanamycin, 7 μg/mL gentamycin, 10 μg/mL tetracycline, 40 μg/mL IPTG, and 100 μg/mL Blue-gal (*see* **Note 2**) and once set, allow to dry, inverted at RT.
2. Using a multichannel pipette, add 3 μL of recombinant DNA to a 96-well PCR plate.
3. On ice, add 30 μL of chemically competent *E. coli* DH10Bac or DH10EMBacY cells using a repeat pipettor (*see* **Note 3**), cover with an adhesive tape pad and incubate for 30 min. It is advisable to include a positive control (i.e., a construct that has previously shown soluble protein expression in BEVS) in position H12 of the 96-well plate.
4. In the meantime, add 900 μL of prewarmed 2× LB medium containing 50 μg/mL kanamycin, 10 μg/mL tetracycline, and 1 μg/mL gentamycin to each well of a 96-deep-well block using a reagent reservoir.
5. Heat shock the cells in the PCR plate for 45 s in a 42 °C water bath and return briefly to ice.
6. Transfer the bacterial suspension into the prewarmed medium block (**step 4**), cover with a porous seal and incubate in a Glas-Col shaker (or equivalent) at 37 °C with shaking at 700 rpm for 5 h.
7. Dilute the culture (10 μL into 90 μL) into LB (or 2× LB) medium in a 96-well microtiter plate and spread 50 μL onto the recombinant bacmid selection plates (*see* **Note 4**).
8. Incubate the plates at 37 °C for 48 h, covered with foil (*see* **Note 5**).
9. White colonies contain the recombinant bacmid DNA and the blue ones do not (*see* Fig. 2). To ensure that the colonies are white, divide a selective plate into 6 or 8 sectors using a marker pen and label with the well position (e.g., A1). Pick single colonies, streak to dilution using a sterile loop and incubate at 37 °C overnight.

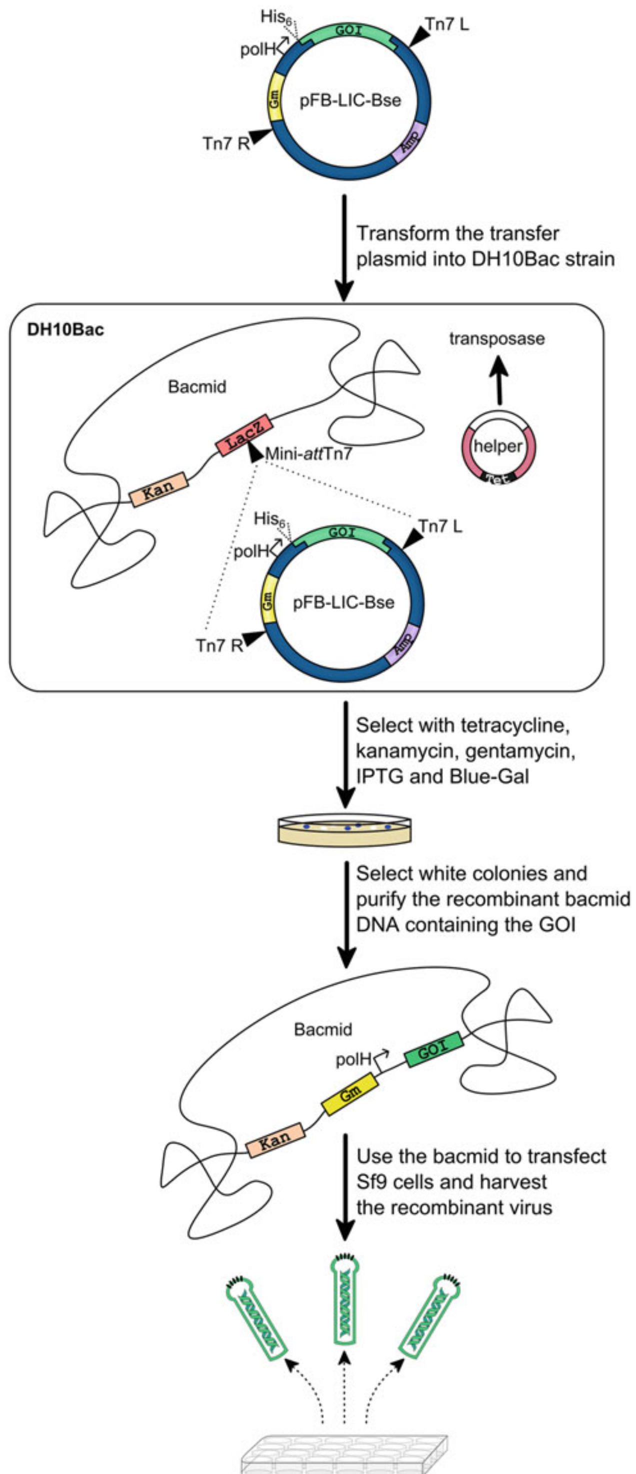

Fig. 2 Diagram describing the transposition process. The construct DNA is transformed into the DH10Bac *E. coli* strain, which contains both bacmid DNA and a helper plasmid. The transposase, expressed from the helper plasmid, will facilitate transfer of the transposable element including the gene of interest (GOI) into the bacmid. The recombinant bacmid DNA can then be purified and used directly to transfect Sf9 insect cells

3.2 Bacmid Production

1. Inoculate the recombinant white colonies (isolated from the restreaked plates) into the corresponding wells of two 96-deep-well blocks, each containing 1 mL of 2× LB medium per well, supplemented with 50 μg/mL kanamycin, 7 μg/mL gentamycin, and 10 μg/mL tetracycline (*see* **Note 6**).
2. Cover with a porous seal and incubate at 37 °C overnight at 700 rpm in a Glas-Col shaker.
3. The following morning, prepare one or two glycerol stocks by mixing 120 μL of the overnight culture and 30 μL of 60% (v/v) glycerol in a 96-well microtiter plate, and store at −80 °C.
4. Centrifuge the deep-well blocks at 3,000 × *g* for 30 min. Decant the supernatant into a suitable container for Virkon decontamination. Invert the blocks and tap gently on absorbent paper.
5. Add 250 μL of the Solution 1 from the 96-well miniprep kit to each well of one block using a multichannel pipette (*see* **Note 7**).
6. Seal the block with a silicone sealing mat (*see* **Note 8**) and mix in the Glas-Col incubator for 2 min at 700 rpm or a 96-well MixMate (or equivalent) at 1,000–1,500 rpm. If necessary, resuspend using a multichannel pipette.
7. Transfer the suspension to the corresponding wells of the second block. Seal and repeat the mixing process.
8. Add 250 μL of Solution 2 to each well, seal with a silicone sealing mat, invert gently 5 times and incubate at RT for 10 min.
9. Add 300 μL of Solution 3, seal with a silicone sealing mat and mix gently but thoroughly by inverting 5 times.
10. Place the sample on ice for 20 min, then centrifuge at 3,000 × *g* for 30 min at 4 °C.
11. Transfer the clear supernatant to a fresh 96-deep-well block, cover with an adhesive tape pad and centrifuge again at 3,000 × *g* for 30 min at 4 °C (*see* **Note 9**).
12. In another fresh 96-deep-well block, dispense 0.8 mL of isopropanol into each well and add 0.8 mL of the clarified supernatant to the corresponding wells (*see* **Note 10**).
13. Using a 1 mL capacity multichannel pipette, gently mix up and down, cover with an adhesive tape pad and then incubate on ice for 30 min (*see* **Note 11**).
14. Centrifuge at 3,000 × *g* for 30 min at 4 °C.
15. Spray the outside of the 96-deep-well block with 70% (v/v) ethanol (*see* **Note 12**) and inside the biological safety cabinet (BSC), remove the cover from the block and discard the supernatant by decanting into a suitable container and blotting on absorbent paper.

16. Add 500 μL of 70% (v/v) ethanol to each well and tap the block gently to wash the pellets. Cover with an adhesive tape pad and then centrifuge at 3,000 × *g* for 30 min at 4 °C.
17. Inside the BSC, open the block and discard the supernatant by decanting. Tap the block very gently on absorbent paper to remove the ethanol. Allow the block to dry inside the hood for approximately 2 h or cover with porous seal and leave overnight in the BSC with it switched on (*see* **Note 13**).
18. Inside the BSC, add 50 μL of sterile TE buffer, cover with an adhesive tape pad and allow to stand for about 1 h. Very gently resuspend the bacmid DNA using a multichannel pipette (*see* **Note 14**) and transfer to a 96-well microtiter plate. Remove a couple of microliters of DNA from a few wells to measure the concentration using a UV-spectrophotometer. Pipette 1 μL of each DNA into a PCR plate for the bacmid PCR screen, then seal with a fresh adhesive tape pad.
19. Store bacmid DNA at 4 °C until the test purification is complete, then store at -20 °C.

3.3 Bacmid PCR Screen

1. Prepare a 10 μM primer stock (50 μL each of the 100 μM forward and reverse primers added to 400 μL of molecular biology grade water) of the bacmid screening primers (*see* Table 1). Store at −20 °C.
2. Dilute the bacmid DNA 1 in 50 in molecular biology grade water in a 96-well PCR plate (*see* **Note 15**).
3. Set up a PCR master mix as follows: 400 μL of 5× MyTaq™ Reaction Buffer Red, 1.49 mL of water, 100 μL of 10 μM of bacmid screening primers (**step 1**) and 10 μL of MyTaq™ DNA Polymerase (5 unit/μL). Using a repeat pipettor or a multichannel, pipette 20 μL into each well of a 96-well PCR plate.
4. Transfer 2 μL of the diluted bacmid (**step 2**) to the PCR plate (**step 3**) and mix well.
5. Seal the PCR reaction plate with an adhesive PCR seal and set a thermocycler with the following conditions making sure that the block is up to 95 °C before placing your sample plate in the instrument:

   95 °C, 5 min

   (95 °C, 45 s; 50 °C, 45 s; 72 °C, 2–5* min) ×25 cycles

   72 °C, 7 min

   15 °C hold

   *Extension time dependent on length of PCR product—for example, 30 s per 1 kb. Please note that additional base pairs will be added to your products due to the positioning of the screening primers (*see* Table 1).

Table 1
Primers used to confirm correct insertions at the bacmid PCR screen stage

Primer Name	Primer Sequence
Fbac-1	TATTCATACCGTCCCACCA
M13bac_rev	CAGGAAACAGCTATGAC

Fbac-1 and M13bac_rev are used for the Baculovirus vectors

6. While the PCR cycle is running, prepare a 96-well 1.5% TAE-agarose gel.
7. Using a multichannel pipette, load 10 μL of the PCR reaction mixtures directly onto the gel. Note that the spacing of the wells means that samples will be interleaved. Load 6 μL of 1 kb Plus DNA Ladder and run the gel at 150 V for 1 h.
8. Confirm the sizing of the products and repeat the screen for any constructs that do not produce a band of the correct size in the first screen (*see* **Note 15**).

3.4 Growth and Maintenance of Insect Cell Lines

Insect cell lines can be maintained in adherent culture as well as in suspension culture. Their ability to grow in suspension at high densities allows for expression of recombinant proteins in large scale; however, their ability to grow in monolayers can be utilized for the initial stage of transfection to generate baculoviruses. The most widely used insect cell lines for BEVS-based protein expression are Sf9, Sf21, and High Five, all of which are adaptable to serum-free, protein-free medium. We routinely use Sf9 cells for all the steps from transfection to large scale protein expression simply because of their robustness and ease in manipulation; however, occasionally High Five cells are used for large scale expression of proteins. Use of Sf9 cells for all steps in routine protocols ensures that uniform parameters are applied to a number of protein targets initially and if needed other cell lines can be tested later on to improve protein expression. Insect cell culture methods are described previously in detail [6, 12, 13]. Some important points when working with insect cells are mentioned in **Note 16**.

3.5 Reviving Sf9 Cell Line from Frozen Stock

Sf9 cells can be revived straight into suspension culture without first reviving them into adherent culture, provided there are sufficient cryovials of cells available in liquid nitrogen. Alternatively revive cells into adherent culture using T-flasks, then transfer to suspension culture at a density of 1×10^6 cells/mL from 70% to 80% confluent flasks, using sloughing off method (i.e., washing off layers of cells, instead of using traditional dislodging methods such as trypsin solution). Cells can be kept in suspension culture for 6–8 weeks, after which time a new stock should be revived as

older cells may show a decline in protein expression. There are different commercial formulations of serum-free insect cell media available; however, we use Sf-900™ II SFM mainly for initial revival of cells, transfection, expression testing, virus amplification and large-scale protein expression. Insect-XPRESS can also be used for large-scale protein expression. Sf9 cells adapt quickly from one medium to another. All of the cell culture steps described below are performed in aseptic conditions inside a BSC.

1. Warm Sf-900™ II SFM medium to 27 °C in a water bath and pipette 30 mL of the medium into a 250 mL flask.
2. Remove a cryovial containing the cells (at 3×10^7 cells/mL) from liquid nitrogen and carefully release the cap to depressurize it, then tighten it (*see* **Note 17**).
3. Transfer the cryovial to a container with warm water (25–30 °C) and incubate until the sample is 70% thawed.
4. Decontaminate the outside of the vial by wiping with 70% (v/v) ethanol.
5. Using a 5 mL Stripette, transfer the thawed cells immediately into the 250 mL flask containing the medium and pour the remaining icy cells from the cryovial straight into the flask.
6. Gently mix the cell suspension and transfer the flask to a 27 °C incubator with shaking at 90–100 rpm.
7. Check the cells after 48 h for good health.

3.6 Suspension Culture of Sf9 Cells in Shake Flask

Cells previously cultured in an anchorage-dependent manner need complete adaptation to suspension culture. The cells can be grown in suspension using either shake flasks or spinner flasks; however, our method of choice is the former. The use of simple shake flasks makes the process of protein expression in insect cells easily scalable from 10 mL to more than 10 L volume and does not require specialized equipment, which would be needed for spinner flasks and bioreactors.

1. After growing a sufficient number of cells, determine the viable cell count using Trypan Blue Stain and a hemocytometer (*see* **Note 18**).
2. Seed the cells to a density of 1×10^6 cells/mL into a 500 mL nonbaffled polycarbonate or glass flask in Sf-900™ II SFM medium.
3. Incubate the flask at 27 °C with shaking set at 90–105 rpm (*see* **Note 19**).
4. When the cells reach a density of 4×10^6 cells/mL, dilute them back to 1×10^6 cells/mL and expand the cell volume depending on requirement of the cells (*see* **Note 20**).

3.7 Cell Freezing

Once the cells start doubling regularly after revival it is advisable to freeze down the low passage number cells in several cryovials.

1. Prepare freezing medium containing 92.5% (v/v) Sf-900™ II SFM medium and 7.5% (v/v) DMSO and store at 4 °C.
2. Label sterile cryovials with the name of the cell line, date of freezing and any other relevant information and store the vials at 4 °C until ready to use.
3. Take a small suspension of cells from a shake flask and count viable cells using a hemocytometer. Alternatively, cells from adherent cultures can be used for freezing.
4. Take the required volume of cell suspension for 3×10^7 cells per vial.
5. Centrifuge the cells at $500 \times g$ for 10 min and discard the supernatant.
6. Resuspend the cells in the freezing medium (prepared in **step 1**) so that after resuspension the cell density is ~3×10^7 cells/mL.
7. Quickly aliquot 1 mL of the cell suspension into the cryovials (prepared in **step 2**).
8. Place the vials into a suitable freezing container (e.g., Mr. Frosty) and transfer the container to a −80 °C freezer overnight (*see* **Note 21**).
9. The following day transfer the vials to liquid nitrogen storage.

3.8 Decontamination and Cleaning of Shake Flasks

It is extremely important to clean the shake flasks properly so that they can be reused without affecting the cell health or cell growth. Any residual disinfectant or scum of dead cells can adversely affect the cells and protein expression.

1. Pour off any spent media into a waste container and add 1 tablet of Virkon per L of the spent media.
2. Completely fill the empty culture flask with a 1 in 200 dilution of Chemgene and leave for at least 20 min (but no longer than 30 min). Make sure that every surface of the flask that has come in contact with virus is covered with the diluted Chemgene (*see* **Note 22).**
3. Discard the decontaminated waste and rinse with tap water.
4. Add a scoopful of Alconox®, fill the flask with water, incubate for 20 min and scrub with a laboratory bottle brush to make sure that there is no visible cell debris or dead cell scum remaining inside the flask.
5. Leave the flask with fresh water for minimum 1 h.

6. If available, wash the flasks using a washer-disinfector according to the manufacturer's instructions.
7. Dry the flasks in a drying cabinet set at 50–60 °C, cover with two layers of aluminum foil and autoclave.

3.9 Transfection into Sf9 Cells

1. Prepare ~100 mL of Sf9 cells 1 day in advance by diluting the cell count to 1×10^6 cells/mL in Sf-900™ II SFM medium.
2. The next day dilute the mid-log phase Sf9 cells to 2×10^5 cells/mL in Sf-900™ II SFM medium.
3. Label four 24-well tissue culture (TC) plates with 'plate 1' to 'plate 4' to cover your 96 samples (*see* Fig. 3 for how to transfer samples between 96-well and 24-well blocks or plates).
4. Using a 1 mL 12-channel multichannel pipette (with 6 tips spaced two apart), dispense 1 mL of diluted culture (**step 2**) into each well of four 24-well TC plates. Include controls: one for Insect GeneJuice®-only and the other for untreated cells (*see* **Note 23**). Incubate the plates at 27 °C for 1 h to allow for cell attachment (*see* **Note 24**).
5. Mix 200 μL of Insect GeneJuice® with 4 mL of Sf-900™ II SFM medium in a sterile 15 mL tube (sufficient for 100 reaction wells). Gently vortex for 10 s.
6. Dispense 40 μL of the mixture prepared in **step 5** into a sterile 96-well microtiter plate (leaving a well empty for the cell-only control).
7. Transfer 2 μL of recombinant bacmid DNA (concentration should be 0.5–2 μg/μL) into each well and cover the microtiter plate with an adhesive tape pad. Mix by tapping the plate gently or pipetting (*see* **Note 14**).
8. Incubate the mixture inside the BSC for 30 min; this incubation time is critical and extensions should be avoided.
9. After incubation, add 160 μL of Sf-900™ II SFM medium to the mixture in **step 8**.
10. Remove the 24-well TC plates containing the cells (**step 4**) from the incubator and aspirate the medium from the cells using a multichannel pipette.
11. Add the 200 μL DNA–Insect GeneJuice® mixture from **step 9** dropwise onto the cells using a 12-channel multichannel pipette (with 6 tips spaced two apart) following the layout from Fig. 3 (*see* **Note 25**). Gently rock the plates back and forth and from side to side.
12. Incubate the cells for 4 h at 27 °C, in a humidified incubator.
13. Gently add 0.4 mL of Sf-900™ II SFM insect medium containing 2% (v/v) FBS to each well (*see* **Note 25**). Incubate the cells at 27 °C in a static incubator for 3 days.

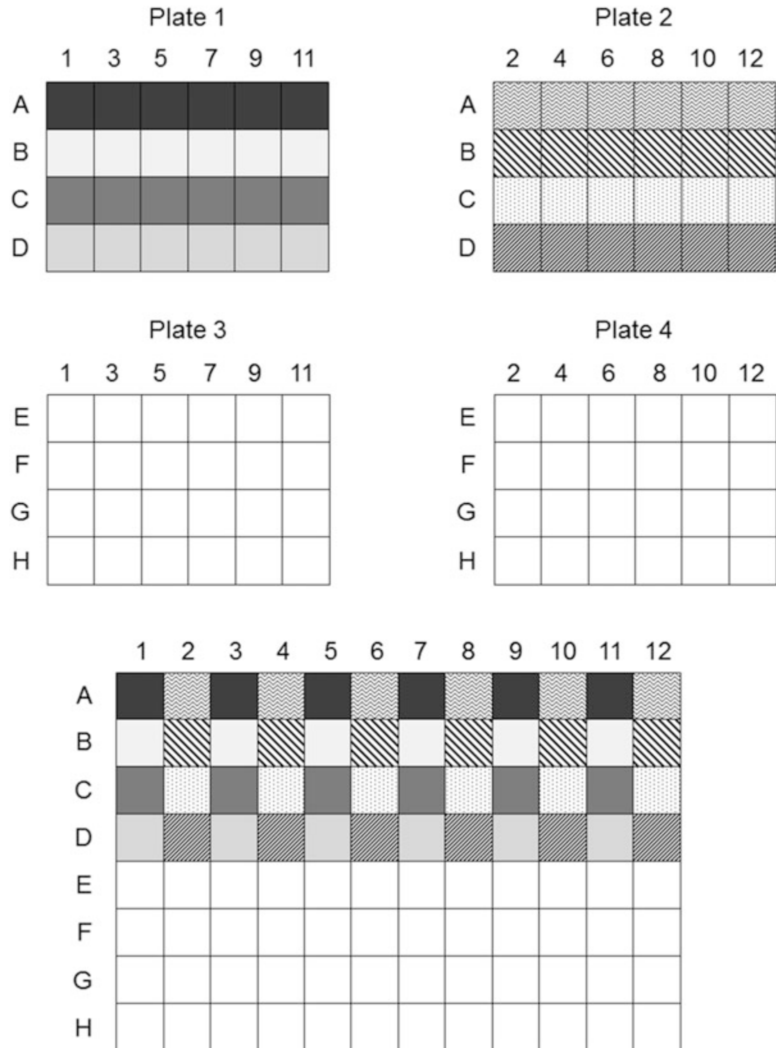

Fig. 3 The format for transferring samples between 24-well and 96-well blocks

14. Signs of infection should be seen in the transfected cells 2–3 days posttransfection, by comparing with the control cells under an inverted microscope. Confluent growth of cells will be seen in control wells, whereas areas of clearing will be prominent in wells with infected cells. Infected cells are usually larger and deformed or elongated compared to uninfected cells.
15. Harvest the viruses when the cells are well infected by transferring the liquid contents from the 24-well TC plate into a sterile 96-deep-well block (*see* Fig. 3 for layout) and centrifuging at 1,500 × *g* for 20 min at RT. Collect the clear supernatant (<0.7 mL) in another sterile 96-deep-well block. This is the P0 baculovirus (BV) stock, which is stored at 4 °C, protected from light.

3.10 Virus Amplification and Test Expression

1. Using a 1 mL multichannel pipette, dispense 3 mL of Sf9 cells (in Sf-900™ II SFM medium, containing 2% (v/v) FBS, at a density of 2×10^6 cells/mL) into each well of four 24-deep-well blocks.
2. Following the layout shown in Fig. 3, infect the cells with 120 μL of P0 BV stock (*see* **Note 26**) and incubate at 27 °C, with shaking at 450 rpm in a Glas-Col shaker for 66–72 h (i.e., set up late on day 1 and harvest early on day 4).
3. Pellet the cells by centrifugation at 1,500 × *g* for 20 min and harvest the supernatant by pipetting into a 96-deep-well block in the BSC according to the layout shown in Fig. 3. Store as P1 BV stock at 4 °C in the dark.
4. Resuspend the pellets in 1 mL of Lysis buffer, supplemented with protease inhibitors, and store at −80 °C for test purification at later date (or preferably purify directly).

3.11 Test Purification

1. If frozen, thaw pellets in a water bath at RT, then sonicate on ice for 4 min (3 s on, 15 s off with 35% amplitude on a 750 watt sonicator) using a 24-head probe (check that the probe is level and all tips are in the liquid; after sonication check for clearing).
2. Remove 15 μL of the total cell lysate into a 96-well PCR plate as the Total fraction, add 5 μL of the 4× sample buffer, and store at 4 °C.
3. Transfer the remaining sample into a 96-deep-well block according to the layout shown in Fig. 3 and centrifuge at 3,000 × *g* for 30 min at 4 °C.
4. Remove the clarified supernatant to a fresh 96-deep-well block using a multichannel pipette, taking care to avoid transferring any pelleted material (*see* **Note 27**).
5. Add 100 μL of a previously washed and equilibrated 50% slurry (Ni-IDA or Ni-NTA) to each well using a multichannel pipette with cut tips, mixing well before each row (*see* **Note 28**).
6. Seal the block with a silicone mat and place another 96-deep-well block on top, tape together and incubate at 18 °C on a rotating wheel for 1 h, spinning at 10 rpm (*see* **Note 29**).
7. Centrifuge the block for 30 s at 200 × *g* to remove the liquid from the lid and load the mixture on to a 96-well filter plate placed on top of a 96-deep-well waste collection block.
8. Allow the liquid to drip through the filter plate or centrifuge at 200 × *g* for 1 min.
9. Add 800 μL of Wash buffer to the resin block to wash out the remaining resin and then transfer to the corresponding wells of the filter plate. Allow the buffer to flow through or centrifuge briefly at 200 × *g*. Pour off the buffer from the waste block after this and all subsequent washing steps.

10. Add 800 μL of Wash buffer and allow the buffer to flow through or centrifuge briefly at 200 × *g*.
11. Repeat the wash step a further 3 times and after the final wash, spin the plate for 2 min at 300 × *g* to remove any residual Wash buffer. Pour off Wash buffer from the waste block and spin for a further 1 min to remove all trace of Wash buffer (*see* **Note 30**).
12. Place the filter plate on top of a fresh 200 μL V-bottomed 96-well microtiter plate and add 50 μL of Elution buffer to each filter well.
13. Incubate at RT with shaking for 20 min, then centrifuge for 3 min at 300 × *g* to collect the elution (Purified fraction).
14. In a 96-well PCR plate, mix 15 μL of each Purified fraction with 5 μL of 4× sample buffer. Heat denature at 80 °C for 10 min.
15. Prepare four SDS-PAGE precast gels by rinsing with water, adding 1× MES buffer and rinsing the wells.
16. Using a multichannel pipette, load 15 μL of your samples onto the gels, note that samples will be interleaved (e.g., A1, B1, A2, B2, etc.). Also load 5 μL of a protein marker (e.g., SeeBlue® Plus2 Pre-Stained Standard) in the first lane of the gel.
17. Run the gel at 150 V for at least 1 h, or as long as required for the dye-front to reach the bottom of the gel.
18. Break open the cast and carefully remove the gel into a tray, rinse with water, and add half a cap full of InstantBlue™. Stain for ~1 h with shaking at RT.
19. Discard the stain and wash twice with water, taking care not to tear the gel. Leave in water with shaking to destain for as long as required.
20. Confirm the size of your protein of interest against the protein ladder (*see* **Note 31** and Fig. 4).

3.12 Virus Amplification

The volumes of P0 (0.7 mL) and P1 (3 mL) viruses generated as described previously are low in volume and insufficient to be used for large-scale expression experiments. Therefore, it is necessary to amplify the virus in a larger volume, typically to the scale of 50–100 mL. The virus can be stored at 4 °C for months, but it is advisable to reamplify the virus, if stored at 4 °C for a longer period of time. For virus amplification, insect cells are generally infected with low Multiplicity of Infection (MOI—number of virus particles per cell) to avoid generating noninfectious particles in the virus stocks. Use a healthy log phase culture of Sf9 cells with more than 95% viability. All of our virus stocks are made in Sf-900™ II SFM, but other media formulations may work equally well.

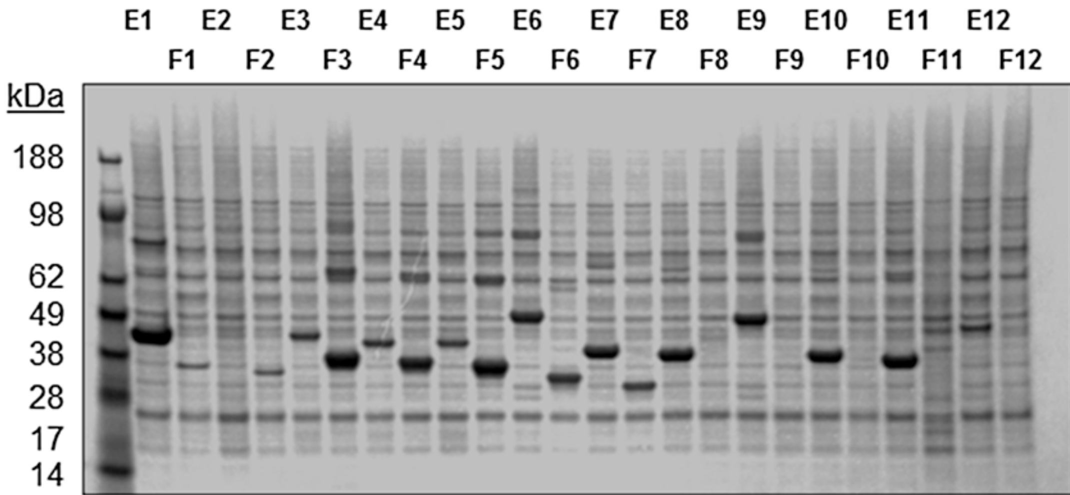

Fig. 4 Image showing the SDS-PAGE result of a test purification from insect cells. The gel shows a range of high, medium, and low expressions of various proteins of different molecular weights. Note that samples loaded using a multichannel pipette will be interleaved (e.g., A1, B1, A2, B2, etc.)

1. Take a sterile 250 mL or 500 mL flask and seed 50 mL of suspension-adapted Sf9 cells (2×10^6 cells/mL) in Sf-900™ II SFM.
2. Add FBS to the final concentration of 2% (*see* **Note 32**).
3. Add 100 μL of the P1 BV stock to the cells and gently swirl the flask.
4. Transfer the flask to a 27 °C shaking incubator with shaking speed set at 100 rpm and incubate the flask for 72 h.
5. At 72 h postinfection take a small aliquot of cells and observe under the microscope for signs of infection (*see* **Note 33**) and absence of any form of microbial contamination.
6. Transfer the cells to a 50 mL tube and centrifuge at $900 \times g$ for 20 min.
7. Collect the supernatant into a fresh 50 mL tube and store at 4 °C. This represents P2 BV stock.
8. The cell pellet generated in the process of virus amplification can be utilized for protein purification using IMAC. Protein purified from this pellet can be used for any intended application. Moreover, this purification validates the ability of the virus stock to express protein.

3.13 Large-Scale Expression

This protocol is successfully applied for the expression of a broad range of proteins but for some proteins the expression time point, and MOI can be highly specific and will require optimization (*see* **Note 34**).

1. Seed log phase Sf9 cells to the density of 1×10^6 cells/mL in Insect-XPRESS or Sf-900™ II SFM. Keep the volume of culture to 1 L in a 3 L capacity flask. If more than 1 L scale-up is needed, use multiple 3 L flasks with 1 L culture volume in each (*see* **Note 35**).
2. Incubate flasks at 27 °C with shaking set at 100 rpm and allow the cells to grow for 24 h.
3. The next day, check the cell density using a hemocytometer and cell health and look for any signs of contamination. Cells should go through one doubling cycle in 24 h and the cell count should be ~2×10^6 cell/mL.
4. Add 1.5–3.0 mL of P2 BV stock per L of the culture, swirl the flask gently, and transfer the culture to a 27 °C shaker-incubator set at 100 rpm (*see* **Note 36).**
5. Incubate the flask for 64–72 h.
6. Take a small sample of the infected culture and look under the microscope for signs of infection, but not lysis of the cells. In addition, look carefully for the absence of any bacterial or fungal contamination (*see* **Note 37).**
7. Take 3 mL out of the culture and centrifuge separately (at $900 \times g$ for 20 min) from the remaining culture for expression testing (*see* **Note 38**).
8. Without waiting for results from **step 7** above, transfer the remaining cells to 1 L centrifuge pots, balance pairwise and centrifuge at $900 \times g$ for 20 min using JLA 8.1000 rotor on Avanti J-20XP or Avanti J-26XP centrifuge (*see* **Note 39**).
9. Pour the supernatant to a waste container for decontamination using Virkon.
10. Resuspend the cell pellet obtained from 1 L of the culture in 25–30 mL of PBS by swirling and pipetting gently and transfer to 50 mL tubes.
11. Balance the tubes pairwise and centrifuge at $900 \times g$ for 20 min using a benchtop centrifuge.
12. Discard the PBS in a Virkon solution and proceed to purify the protein from the cell pellet or freeze the cell pellets at −80 °C for purification at a later date.

3.14 Protein Extraction

All the following steps of protein extraction and purification are performed at 4 °C or on ice. Prechill the buffers and centrifuges.

1. If protein purification is performed straight after harvesting the cells, transfer the cell pellets to ice or if the cells were frozen, thaw the pellets in a water bath set at RT or 37 °C. Do not leave pellets in the water bath for any longer than is required to thaw them and transfer onto ice immediately once thawed.

2. Resuspend the cells in 1 volume of ice cold 2× Lysis buffer (1 mL per g wet-weight of cells) using a pipette and add additional Lysis buffer until the suspension is homogeneous.
3. Place the cell suspension container on ice. Set the amplitude to 35% on a 750 watt Sonics Vibra-Cell sonicator and sonicate with 10–15 bursts of 5 s on, 10 s off (*see* **Note 40**). Save 10 μL of the lysate which represents the Total fraction.
4. Transfer the lysates to centrifuge tubes, balance the tubes pairwise, and centrifuge at 53,000 × *g* using a JA-25.50 rotor for at least 30 min at 4 °C.
5. Transfer the clear supernatant into a clean tube taking care to avoid transferring any pelleted material. This clarified supernatant represents the Soluble fraction.

3.15 Large-Scale Protein Purification

The protein purification scheme for insect cells is similar to protein purification from *E. coli* as described in Chapter 4, Subheading 3.6. However, we recommend paying particular attention to the following points while purifying proteins from insect cells:

1. The buffer composition described here works for a diverse set of proteins but the buffer can be substituted to address issues such as protein instability and requirements of final applications. Careful optimization of the buffer composition with respect to the buffering system, pH, salt concentrations, and additives is particularly critical for difficult to purify proteins.
2. In comparison to *E. coli* cell lysates, insect cell lysates are denser because of higher background protein concentration. This can result in clogging of prepacked IMAC columns; therefore, we recommend doing manual IMAC using the gravity-flow procedure for purification of proteins from insect cells.
3. Often intrinsic proteins from insect cells copurify due to the affinity of exposed histidines or metal binding moieties of endogenous proteins toward the immobilized metal ions. Therefore, it is often the case that IMAC followed by SEC is not enough to obtain very pure protein from insect cells, which necessitates inclusion of additional purification steps such as ion exchange chromatography or tag cleavage and rebinding to IMAC.

3.16 Quality Assurance

If available, mass spectrometric analysis of every purified protein is highly recommended. This confirms the molecular weight of the protein, with mass discrepancies indicating mutations or cloning artifacts and potential posttranslational modifications. The protein is loaded into a small C3 HPLC column for desalting and eluted onto an in-line electrospray ionization time-of-flight analyzer. Any discrepancy needs to be explained, either by sequencing the DNA, by enzymatic removal of suspected modifications or by MS/MS analysis of proteolytic fragments.

4 Notes

1. The EMBacY backbone contains a constitutively expressing YFP expression cassette that allows for easy monitoring of viral titers via fluorescence without plaque assays.
2. X-gal does not produce sufficiently dark blue nonrecombinant colonies in our hands; therefore, we use Blue-gal instead. The plates can be stored for up to 1 month at 4 °C, covered with foil to prevent exposure to light.
3. Be careful not to splash the cells against the sides of the wells while using the repeat pipettor and check that the liquid is at the bottom of the well before continuing. This step can also be done using a single channel pipette but will take more time.
4. When there are no colonies, plate 50 μL of undiluted culture instead.
5. This step can be performed at RT on the bench over the weekend if necessary.
6. One 96-well block should provide sufficient bacmid DNA for transfection. However, we find it useful to set up two blocks to provide a balance for the centrifugation step.
7. We only use the reagents from the Montage Plasmid Miniprep$_{HTS}$ 96 Kit for purifying the recombinant bacmid DNA, not the filter plates. The reagents can also be purchased from Merck individually.
8. Covering the block with an adhesive tape pad or alternative will result in leaking and cross-contamination of wells. Make sure the silicone sealing mats are suitable for either round or square 96-deep-well blocks, depending on which 96-well blocks you use.
9. This second centrifugation step is important to remove as much of the insoluble pelleted material as possible in order to obtain clean bacmid DNA at the end of the prep.
10. It is recommended not to remove all of the supernatant to avoid transferring insoluble material.
11. Incubation can also be done overnight at 4 °C and will result in a higher yield of bacmid DNA but is not necessary.
12. If you have more than 1 block, be careful not to remove the marker labels when using 70% (v/v) ethanol.
13. Do not allow the pellets to dry out completely.
14. The bacmid DNA is very fragile so mix it gently, do not over-pipette. If the concentration of the DNA is less than 0.5 μg/μ L, use up to 5 μL.

15. High concentrations of bacmid DNA will inhibit the bacmid PCR screen so we dilute the bacmid prior to addition. Where the yields of bacmid are low it may be necessary to use a lower dilution instead.
16. All cell culture steps must be performed under aseptic conditions in a BSC, making sure that sterility is maintained throughout the procedures. To keep the cultures free from contamination by bacteria, yeast, fungi and viruses, it is crucially important to keep the benches, BSC and incubators clean. Use 70% (v/v) ethanol to wipe the cabinet before and after use, also wipe the outside of media bottles, pipettors, flasks and other containers with 70% (v/v) ethanol before transferring them into the cabinet. Wear clean lab coats and gloves and wash hands before and after working with cell culture. Any spillage inside the BSC, incubators, and so on should also be cleaned immediately with 70% (v/v) ethanol or MicroSol. Use separate media bottles for general cell culture maintenance and for virus work. We recommend adding penicillin and streptomycin to the final concentration of 50 units/mL and 50 μg/mL respectively to the cell culture media to prevent bacterial contamination during culture growth.
17. Always wear protective clothing (lab coat, gloves, and safety specs) when thawing vials containing frozen cells as they sometimes explode on contact with the water. Do not dilute cells below 1×10^6/mL. Final DMSO concentration in suspension should not exceed 0.5%.
18. The % Cell viability is calculated by counting the number of viable cells and also the number of total cells on the hemocytometer grid. Viable cells do not take up Trypan Blue Stain; however, nonviable cells take up the stain and appear blue under the microscope. To determine cell viability, mix 0.1 mL of Trypan Blue Stain with 1 mL of cell suspension and load a hemocytometer. Count the number of blue-stained cells and the total number of cells and then calculate the number of viable cells per mL and correct for the dilution factor. Cell viability should be at least 95% for a healthy log phase culture before it can be used for transfection, virus amplification or protein expression.
19. For better aeration of the cells, it is important to keep the culture volume between 25% and 35% of the total volume capacity of shake flask and shaking between 90 and 105 rpm. Cells form clumps initially but cells should start growing in single cell suspension within a week or so.
20. Cells can be transferred gradually to 1 L and then 3 L flasks, keeping the culture volume between 25% and 35% of the total volume capacity of shake flask. Ideally do not allow the cell

density to exceed 6×10^6 cells/mL or fall below 0.7×10^6 cells/mL. Cell growth may slow down if diluted to the density of less than 0.7×10^6 cells/mL. Cells should not be diluted by more than 1 in 5.

21. If a freezing container is not available, vials can be transferred to a −20 °C freezer for 2–3 h followed by transfer to −80 °C overnight.
22. It is not necessary to keep Chemgene solution in flasks for more than 20 min. Leaving Chemgene for longer may make it difficult to remove the traces from flasks. Glass flasks are easier to clean than the polycarbonate flasks. Polycarbonate flasks for suspension culture are meant to be disposable but they can be reused several times if cleaned properly after treatment.
23. To keep it cost effective and to express most of our recombinant proteins, we tested and compared a range of transfection reagents and decided to use Insect GeneJuice®.

    The Insect GeneJuice®- and cell-only controls are important for determining the success of the transfection as they allow the user to distinguish cytotoxic effects and uninfected cells from infected cells.
24. Cell attachment can be observed using an inverted microscope by focusing through the sample; the cells should be visible in one plane of view once successfully attached.
25. Pipette the mixture gently and avoid touching the bottom of the plate so as to not disturb the cells.
26. For some targets it may be necessary to use the P1 virus to infect for test expression. However, we have found that there is little difference in the yields when expressing from P1 rather than P0. We therefore use P0 virus, which shortens the expression process by at least 3 days.
27. To avoid disturbing the Insoluble fraction, tilt the plate and drive the tips down the side of the wells at an angle. Stop just above the pellet, on most plates there is a ridge just off the bottom—feel for this with the tips. Gently pipette up the supernatant and then transfer to the new plate. Do not go back into the wells as this will resuspend the pellets; if this happens then respin the sample and try again.
28. The resin tends to clump and settles quickly. We recommend using 200 μL tips with ~5 mm cut from the ends to prevent clogging the tips and ensure even loading. Also, continually mix the resin by pipetting up and down as well as shake the reservoir from side to side to prevent settling.
29. When the silicone matting seal is pressed down firmly and held in place with another deep-well block, the block will not leak

when placed on its side. If you prefer you can incubate the plate upright, but the resin tends not to mix as well when done this way; we would therefore recommend keeping the samples in a 24-well format for this step, as this provides greater surface area for binding.

30. Removing all trace of Wash buffer is essential to ensure that the subsequent elution step does not become diluted with Wash buffer.
31. It is beneficial to grade the expression level of your proteins to more easily identify ones that you may wish to scale up. At this point we also recommend confirming the targets using quality control steps such as intact mass (if quantities are sufficient) or by in-gel tryptic digest MSMS analysis.
32. Baculovirus stability is known to improve in the presence of FBS. As Sf-900™ II SFM is a serum-free and protein-free medium, addition of FBS to the final concentration of 2% is recommended to stabilize the virus and maintain its infectivity when it is stored at 4 °C.
33. Signs of baculovirus infection: baculovirus infected insect cells look swollen, nuclei appear to fill the cells and the cells do not show any clumps when compared to a healthy cell control. If the cells are in very late phase of infection, they will start to lyse.
34. Availability of healthy viable cells is very important for successful scale up of a broad range of targets. Culture conditions such as temperature, pH, dissolved oxygen, osmolality, and nutrient composition of the culture medium can influence the infection of the insect cells. In addition, factors such as cell line, expression time point, MOI, and cell density at the time of infection can have significant effects on protein expression in insect cells. This protocol is generically applied to large number of proteins; however, occasionally for some proteins, optimization at protein expression level is necessary to improve the results. Optimization experiments should be performed on a small scale initially and can be later applied to large-scale expressions. The following conditions could be tested for expression optimization: range of MOI, two harvesting time points (48 and 72 h), two cell lines (Sf9 and High Five), or different cell densities (2×10^6 cells mL and 4×10^6 cells/mL). It should be noted that baculoviruses are lytic viruses for insect cells and will eventually lyse the cells if left long enough after infection. This also means that a harvesting time of 48 or 72 h is also determined by the volume of virus added. The cells can be infected with low MOI (0.05–0.3 pfu/cell) and harvested at 72 h or they can be infected with a high MOI (>1 pfu/cell) and harvested at 48 h. Cells infected with high MOI and harvested at 72 h may show significant lysis.

35. Before diluting the cells, check for the health of the cells and absence of any signs of infection or contamination under a microscope. If less than 1 L scale-up is enough, smaller flasks should be used. However, remember to use a culture volume of only 25–35% of the total volume capacity of the flask.
36. The amount of virus added is determined by the titer of virus stock. We do not routinely measure viral titers but various methods for baculovirus titration have been developed based on cell viability, plaque formation, antibody-based assays, and so on [14]. For the 72 h expression time point, we recommend an MOI of 0.05–0.3 pfu/cell. If the titer of virus stock is 1×10^8 pfu/mL and 2 mL of virus is added to 1 L of the cells (total of 2×10^9 cells), that would be an MOI of 0.1. Addition of more virus can affect the expression and can also cause cell lysis.
37. It should be noted that good signs of infection are desirable but more than 10% lysis of cells can be detrimental to the protein purification.
38. This small volume of cells can be used for expression testing before committing to purify a large batch of cells. This can give a quick estimate of protein expression levels or any failure of the batch to express the protein of interest. To purify the protein from 3 mL of culture, follow the protocol as described in Subheading 3.11.
39. Sf9 cells become very fragile after infection and can rupture if centrifuged at very high speed resulting in loss of protein in the medium itself. We recommend harvesting the cells by centrifugation at $900 \times g$ for 20 min and handling cell pellets gently.
40. Sonication time may need to be adjusted depending on volume of the cell suspension. Avoid excessive foaming and heating of the suspension by adjusting the instrument settings and keeping the cell suspension on ice all the time to reduce the potential for protein precipitation or denaturation. Cell disruption by sonication can also help in reducing viscosity by shearing nucleic acids.

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