Insect cell culture/baculovirus expression

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General information about insect cell culture

There are many advantages to using baculovirus for gene expression, the foremost of which is that baculovirus expression permits folding, post-translational modification and oligomerization in manners that are often identical to those that occur in mammalian cells. These include proper proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, and prenylation. In addition, the insect cytoplasmic environment allows proper folding and S-S bond formation, unlike the reducing environment of the *E. coli* cytoplasm. These advantages make insect cell culture an important tool in the expression of eukaryotic proteins.

Types of cell lines

There are three commercially available cell lines generally used for baculovirus expression: Sf9, Sf21 and High Five. Sf9 and Sf21 cells are very similar and both derive from the pupal ovarian tissue of the fall army worm *Spodoptera frugiperda*. Both cell lines are suitable for transfection, plaque purification, generating high-titer stocks of virus, plaque formation and expression of recombinant proteins. The High Five cell line is derived from ovarian cells of the cabbage looper *Trichoplusia ni*. High Five cells can be used for all of the same experiments, but produce higher levels of recombinant protein than Sf9 or Sf21 cells. Our lab uses Sf9 or High Five cells in monolayer culture for generation of high-titer stocks of virus and High Five cells in suspension for protein expression.

Media

All the insect cell lines that we own have been adapted to serum-free media. For Sf9 cells Sf-900 II SFM is purchased from Invitrogen/Gibco, made 10 µg/mL in Gentamycin and sterile filtered before use. For High Five cells, we use Express Five SFM, also from Gibco. Express Five SFM must be supplemented with 45 mL Glutamine (200 mM) per 450 mL media, and is also made 10 µg/mL in Gentamycin before sterile filtration.

Good sources of information:

Insect cell culture protocols can be found in many places online including:
www.invitrogen.com
http://www.mrw.interscience.wiley.com/cp/cpmb/cpmb_contents_fs.html
(Current Protocols in Molecular Biology Website)

Protocols:
All handling of insect cell lines should be carried out under sterile conditions, as you would for mammalian cell culture. Insect cells are Biohazard Level 1, but are susceptible to bacterial infection if sterile technique is poor.

Cells are maintained at 27-28 °C in both monolayer and suspension culture

CO_2 atmosphere is **not** necessary.

Protocol 1: Thawing cells from frozen stock

1. Pre-warm desired media for 10 minutes in 37 °C water bath

2. Transfer media, 100 mm tissue culture dish, and 25 mL pipette to hood.

3. Transfer 15 mL media into tissue culture dish

4. Remove vial of cells from liquid nitrogen or dry ice (if just received) and place in 37 °C water bath. Gently agitate vial until cells are *almost* thawed, then quickly sterilize vial with ethanol and transfer to hood

5. Transfer the 1 mL cell suspension directly into the 15 mL media

6. Disperse cells by gently rocking the plate side-to-side and forward and back. **Do not rock in a circular motion as this will concentrate cells along rim of the plate.**

7. Transfer plate to a 27 °C incubator and allow cells to attach for 30-45 minutes.

8. After the cells have attached, transfer the dish back to the hood and gently aspirate off the medium. (This removes DMSO leftover from the freezing medium and cellular debris).

9. Add 15 mL fresh medium and transfer back to incubator

10. Change medium again after 24 hours and check cell viability by observing them under a microscope. The majority of cells should look round and healthy, and be attached to the surface (a few floaters are OK, especially with High Five cells, which do not attach as tightly as Sf9)

11. When cells become confluent, passage them into a 150 mm dish.

Protocol 2: Passaging cells (adherent)

Adherent cells should be passaged upon reaching confluency (cells cover the bottom of the dish). Cells should be discarded after about 30 passages (2-3 months).

1. Transfer pre-warmed medium, cells in culture dish, a new dish, and a 10 mL pipette into the hood.

2. Add desired amount of fresh medium into new dish (100 mm = 15 mL, 150 mm = 25 mL)

3. Aspirate medium off cells

4. Add 5 mL fresh medium to cells. Dislodge cells by tilting dish at 45° angle and streaming medium over cells, passing flow back and forth. Repeat until entire bottom of dish is no longer cloudy.

5. *If you are passaging into the same size dish,* add 1.2 mL (~1:4 dilution) of cells to new dish and gently rock back and forth to distribute cells.

If you are increasing dish size (i.e. 100 mm to 150 mm), add all 5 mL to the new dish. This may result in the cells becoming confluent very quickly (24 hrs) in the new dish, but it is better to add too many cells than too few. Passage the cells as described above into a fresh dish of the same size when they become confluent.

Protocol 3: Amplifying virus

Virus amplification can be carried out in either Sf9 or High Five cells. The Koleske lab uses Sf9, but we have had better luck with the High Five cells.

1. Grow a 150 mm adherent cell culture to confluency.

2. Slough off the cells as described in Protocol 2.

3. Count the cells.
4. Add 1.3 x 10^7 cells to a fresh 150 mm plate with 25 mL media.

5. Let cells attach for 1 hour

6. Add 1 mL virus

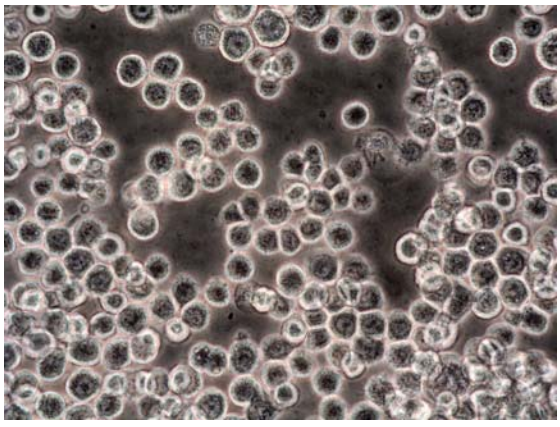
7. Incubate at 27 °C for 6 days. Cells should start to show effect of infection (see below). If not, let the virus go 1-2 days longer.

8. Slough cells and transfer to a 50 mL Eppendorf tube.

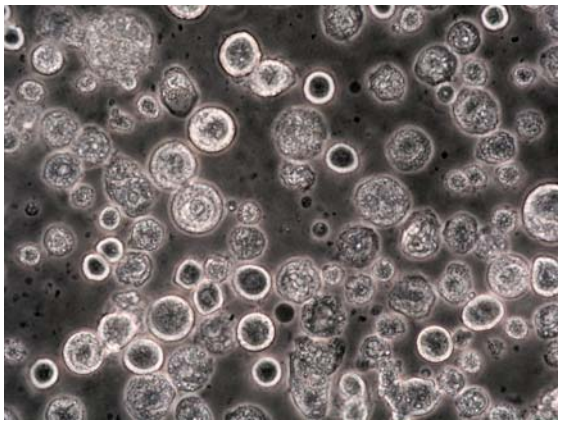
9. Centrifuge 10 min at 1000 x g. Filter supernatant through a 0.45 µm filter. Virus may be stored in the dark at 4 °C for short term storage. Freeze several 1 mL aliquots and store at -80 °C as a backup.

Effects of baculoviral infection on insect cells: Infected cells are larger than uninfected cells and have enlarged nuclei. The cells will stop dividing early in infection and may have a low cell density. Infected cells do not attach very well to the plate, and there may be a large percentage of floaters. Pictures below are under 40x magnification.

Healthy High Five Cells



High Five Cells 6.5 days post infection



Protocol 4: Adaptation of High Five cells to suspension culture

1. Grow four 150 mm tissue culture dishes of High Five cells to confluency

2. Aspirate off supernatant from each dish and slough cells off surface with 5 mL fresh medium

3. Combine cells from all four dishes into a 50 mL Falcon tube.

4. Count cells, keeping in mind that they will probably have to be diluted at least 1:10 to get a reasonable number of cells on the hemocytometer

5. Transfer 1.5 x 10^7 cells to a screw-cap 250 mL Erlenmeyer flask (glass) and add enough fresh media for a final volume of 50 mL. Your final cell density will be 0.3 x 10^6 cells/mL.

A note on glassware: ***Do not clean flasks with detergent*** as even small traces can affect insect cell growth. Instead clean with 10% acetic acid then rinse five times with tap water, and five times with DI water before autoclaving.

6. Transfer to the shaking incubator and incubate at 27 °C, 90 rpm.

7. Count the cells after 24 hours. If the cell density is below 2.0 x 10^6 cells/mL, continue to grow the cells. (Cells usually take 2-3 days to reach this density). Also assess whether the cells look healthy, and if they are forming large aggregates (>10 cells). If at any time the cells begin forming large aggregates, add heparin to the medium at 10 units/mL culture. Cells must be weaned from heparin before using them for protein expression. This is best accomplished by adding medium without heparin when splitting the cells.

8. Passage the cells when their density reaches between 2.0 x 10^6 and 2.5 x 10^6 cells/mL.

Protocol 5: Passaging cells (suspension)

1. Count cells. Passage at cell density between 2.0 x 10^6 and 2.5 x 10^6.

2. Dilute desired number of cells into desired amount of new medium in a fresh flask. Cell density after dilution should be 0.3 x 10^6 cells/mL. For 50 mL of medium, transfer 1.5 x 10^7 cells. For 250 mL medium, transfer 7.5 x 10^7 cells.

3. Place new flask in shaking incubator.

Protocol 6: Expressing protein in High Five cells

1. Grow cells in suspension to a density of 2.0 x 10^6 cells/mL *in a plastic disposable flask*. Using plastic prevents cross-contamination by virus into non-infected cell cultures.

2. For a 50 mL culture, add 1 mL amplified virus. (If protein expression is too low more or less virus can be added and the optimal amount empirically determined).

3. Incubate with shaking for 48 hours.

4. Harvest the cells by spinning in a 50 mL Falcon tube at 800 x g for 10 minutes.

5. Carefully removed the supernatant without disturbing the cell pellet.

6. Cell pellets can be stored at -20 °C for at least several days (possibly more) before lysis.

Protocol 7: Freezing down cells for long-term storage

Cell line	Freezing Medium	Density (cells/mL)
Sf9	60% Grace's Insect Medium, unsupplemented 30% FBS 10%DMSO	1×10^7
High Five	42.5% conditioned Express Five SFM 42.5% fresh Express Five SFM 10% DMSO 5% FBS	3×10^6

1. Count cells. You will need enough cells to freeze down 2-4 cryovials at the densities shown in the above table. Cells can come from suspension or adherent culture.

2. Centrifuge the cells at 400-600 x g for 10 minutes at room temperature. Remove the supernatant. For High Five cells, save the conditioned medium in order to make the freezing medium

3. Resuspend the cells to the given density in the freezing medium indicated

4. Transfer 1 mL of the cell suspension to sterile cryovials

5. Transfer cryovials to Mr. Frosty and place at -80 °C for 24 hours.

6. Store in liquid nitrogen.