Baculovirus/Insect cell expression system for beginners

Introduction

Baculovirus mediated insect cell expression system is one of the most popular eukaryotic expression systems for protein production. This has been a main workhorse to fulfill high demand of recombinant protein needed for structural, biophysics and biochemical studies. The eukaryotic system has been in use for more than 2 decades. Many of the methods developed at inception are still routinely used. Different labs have built up in-house experience over the time to customize their work flow with incremental modifications. This might be the reason the protocol developed in one lab is not easily reproducible in another when it comes to deal with targets that are difficult to express or protein complexes which requires attentive details. Here we tried to share the experience of our lab from last 14-15 years that may be useful for new user of insect cell expression system.

First and foremost the important part we need to consider is how the cells are grown and maintained over the time. We have observed cells maintained in different medium over long period of time has a different properties. It is noteworthy to remember if cells were maintained with a long passage number you may see the superior quality that might be useful for you. This might have happened by clonal selection over the time without our notice.

1. Initiation of Insect cell culture

Decide whether you want to start the culture in monolayer or in suspension. Our choice is growing and maintaining cells in suspension culture. Suspension culture helps to expand cells fast with possibility to keep cells at high density.

Have it ready:
- Incubator shaker (27^{o}C)
- Cell counter (electronic eg. Vi-Cell counter or Neubauer's counting chamber)
- Inverted light microscope
- Laminar flow hood suitable for cell culture.
- Centrifuge
- Consumables (eg. pipette, TC flasks or Erlenmeyer flasks, etc.)
- Insect cell media, Fetal calf Serum (FCS)

Recovery and growth of cells from frozen stock

a. Pre-warm the medium for growth and maintenance of the cells.

b. Take out a vial of Sf9 cell lines from liquid nitrogen and put on dry ice until ready to use.

c. Take 50mL TubeSpin (tube with vented cap) or 125mL Erlenmeyer flask with vented cap and dispense 9mL of pre-warmed medium in it.

d. Recover frozen culture from dry ice and thaw rapidly in a 37^{o}C water bath.

e. Transfer the content to TubeSpin or Erlenmeyer containing 9mL medium.

f. Incubate at 27^{o}C with 170-220 rpm for 4-5h in Bench top incubator (Kuhner, 25mm orbital diameter).

g. After 4h, centrifuge the tubes at 500xg for 5 min.

h. Decant the supernatant and gently re-suspend the pellet in 10 mL of fresh medium.

i. Incubate at 27^{o}C with 220 rpm.

j. Monitor the growth every 24 to 48h.

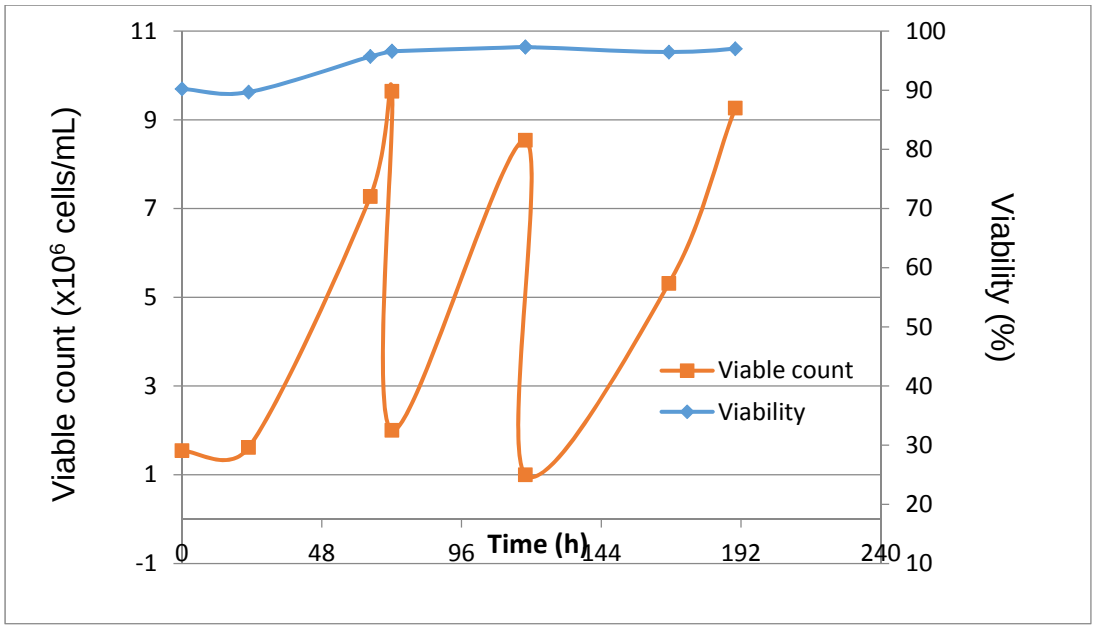


Figure 1a

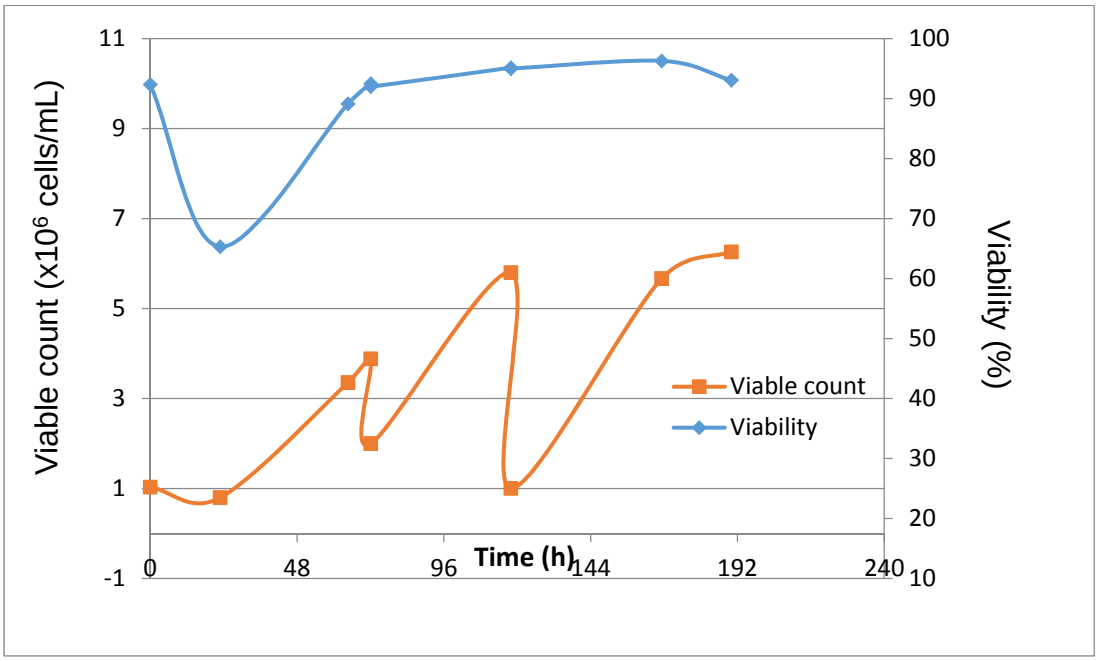


Figure 1b

Figure 1. Growth of Sf9 insect cells in Sf900III medium (1a) and SF4 medium (1b). Once the cells reach near to stationary phase the culture was diluted to about $1x10^6$cells per mL with fresh medium. (Note: viability decreased in case of Sf9 cells grown in SF4 initially and picked up after 48h, this has been routinely observed in SF4 medium)

2. Transfection

The first step in Baculovirus Expression Vector system (BEVS), i.e. maintenance of cell in suspension culture and Transfection, is explained in a method paper recently published by our group (Roest et al., 2016 Transfection of insect cell in suspension for efficient baculovirus generation). The method is based on transfection using PEI. It also covers the amplification of virus required for the production of protein. Main steps involved are extracted and presented here.

2.1 Preparation of polyethyleneimine (PEI)

Have it ready:
- Polyethyleneimine (PEI), Cat no. 23966-2, Polysciences.
- Concentrated hydrochloric acid (12M HCl)
- 10M NaOH
- Vacuum device
- Glassware (beaker and measuring cylinder)
- Magnetic stirrer

This protocol was adapted from "TransientExpressioninHEK293-EBNA1Cells," Chapter12, in Expression Systems (eds. Dyson and Durocher). Scion Publishing Ltd., Oxfordshire, UK, 2007.

a. Take approximately 450mL of Milli-Q water in a 500mL glass beaker.

b. Add 500mg of PEI in a beaker with gentle stirring.

c. Add 12M HCl drop-wise to the solution until the pH drops <2.0.

d. Stir until the PEI is dissolved (~2–3 h). Monitor and maintain pH <2.0 throughout. Approximately 800μL of 12M HCl will be required for full PEI dissolution. There may still be some small fiber-like particles that will not dissolve.

e. Add concentrated NaOH dropwise to bring solution to neutral pH (pH 7.0). Approximately 500μL of 10M NaOH will be required to neutralize the PEI solution.

f. Pour the solution into a 500mL glass cylinder. Adjust the final volume to 500mL with Milli-Q water.

g. Filter sterilize the solution through a 0.22μm membrane filtration using vacuum filtration device.

h. Store aliquots of the desired volume at -20^{o}C.

2.2 Maintenance of cells for transfection

Have it ready:
- Cell culture tube or Erlenmeyer flasks,
- culture media,
- cell counter (manual or automated)

In order to manage the work flow and time, Wednesday is the best time to start transfection in our set up. Hence the procedure is described accordingly. Monday is taken as a Day1 of a week. You can adjust it to fit best for your schedule.

a. Take a small volume of cell culture from master stock and dilute to (0.7–0.8) x 10^6 cells per mL in Sf900III medium on Day5 of a week i.e. on Friday.

b. The following week on Day1 (e.g. Monday), dilute cells to (0.7–0.8) x 10^6 cells per mL in Sf900III medium.

c. On Day2 (i.e. Tuesday) dilute further to (0.7–0.8) x 10^6 cells per mL in fresh medium.

d. On Day3 (i.e. Wednesday), take above culture and dilute to 0.5) x 10^6 cells per mL. Use 1mL of diluted culture per well of 24-deep-well block (Cat.no.7701-5110, Whatman) for each transfection.

2.3 Transfection and virus generation

Have it ready:
- Cell culture grown and maintained in log phase (as described above in Section 2.2)
- Minimal media eg. TC100
- DNA construct in compatible transfer vector
- Bacmid DNA / flashBAC DNA
- Sterile PEI prepared in Section 2.1
- Oven incubator and shaker incubator set at 27^{o}C
- Heat block or PCR machine to heat DNA at 55^{o}C
- 24-well block or 50mL tube for transfection culture

Cloning a gene of interest into appropriate vector is not described here. Various transfer vectors are commercially available. Clone and isolate the construct as per your convenience. We routinely use flashBac system for the transfection hence the same is described here. Same procedure can be used for Bac-to-Bac system.

Preparation of construct DNA

a. Prepare construct DNA at final concentration of 100ng per μL.

b. Heat at 55^{o}C for 1h to avoid possible bacterial contamination.

c. 1.8 μl (180 ng) of it will be used for single transfection.

Preparation of PEI-DNA complex

a. For a single transfection take 360μL of TC100 Medium (Cat.No.T3160,Sigma-Aldrich) and add 1.8 μL of FlashBac DNA (100ng per μL, Oxford Expression Technologies) and 1.8μL of PEI (1mg per mL).For multiple transfections, prepare the master mix and aliquot before adding the DNA construct.

b. Add 1.8 μL (180 ng) of respective DNA construct cloned in FlashBac compatible vectors in it.

c. Mix gently and incubate at room temperature briefly (5-10 min). Longer incubation does not have any adverse effect.

d. Add DNA-PEI complex to cell suspension prepared in Section 2.2. Shake gently for first 5h (150 rpm) then increase the speed to 450 rpm (GlasCol shaker incubator, 3 mm orbital diameter). For incubator with 25 and 50 mm orbital diameter, 250 and 200 rpm, respectively is recommended.

e. After 5 days first generation of virus would be ready. Centrifuge the block at 4000 rpm for 10 min. Separate the clear supernatant and store at 4^{o}C. This serves as V0 virus stock, which will be used to amplify V1 virus.

3. V1 virus amplification

Have it ready (for Section 3 and 4):
- Cell culture grown and maintained in log phase (as described above in Section 2.2)
- Cell culture medium eg. Sf900 III
- Cell culture tube or Erlenmeyer falsks.
- V0 virus
- Cell counter (manual or automated)
- Oven incubator and shaker incubator set at 27^{o}C
- Containing for virus storage (cryo vials or bottle)

a. Use cells grown and maintained in log-phase in Sf900III medium as explained in section 1. Prepare cell suspension to desire density ($0.8x10^{6}$ cells per mL) by adding fresh medium.

b. Dispense 3mL of diluted culture in each well of 24-well block or 50mL vented tubes as per your requirement.

c. Add 0.5mL of V0 virus to it and incubate the culture at 27^{o}C for 48h.

d. Observe the cells under microscope the next day for the sign of infection. If cells have doubled dilute it back to $0.8\text{-}1.0x10^{6}$ cells per mL.

e. Observe the cells again for the sign of infection.

f. Once viability drops down to around 90% with the significant increase in diameter (i.e. 3-4μm) from the time of infection, culture is ready to harvest for virus.

g. Centrifuge the culture at 4000rpm for 10 min. Aseptically transfer the supernatant to two cryovials. Use one as a master virus stock and next as a working virus stock. Store both stocks at 4^{o}C.

4. Amplification of working virus stock

a. Use cells grown and maintained in log-phase in Sf900III medium as explained in section 1. Prepare cell suspension to desire density ($1.0x10^{6}$-$1.2x10^{6}$ cells per mL) by adding fresh medium.

b. For each 100 mL diluted culture use 100 μl of V1 virus. Note: if you need to amplify only 50 mL of diluted culture, it is still recommended to use 100 μL of V1 virus. There is no linear co-relation of culture volume and volume of V1 virus to be used below 100 μL.

c. Incubate at 27^{o}C for 48h. Monitor in between to check the state of infection.

d. Centrifuge at 4000 rpm for 10 min. Transfer the clear supernatant into a clean sterile bottle. This will be the working stock of virus for target protein expression.

5. Medium to large scale expression

Growing insect cells in suspension with high air space in culture vessel is common in most of the lab. We have been following this with the hope that it allows sufficient air for optimal cell growth. For past several years we have been using reagent bottles to run medium and large scale expression. An innovative method that changed the way we run insect cell culture has been described in Insect cell culture in reagent bottle by Rieffel et al., 2014.

Have it ready:

- Cell culture grown and maintained in log phase (as described above in Section 2.2)
- Cell culture medium eg. Sf900 III
- VITLAB reagent bottles (Cat. No. 100389, VITLAB) and BOLA caps (Article no. H 999-45, Bola) for cell culture.
- AirPore tape sheet (Cat. No. 19571, Qiagen)
- Cell counter (manual or automated)
- Oven incubator and shaker incubator set at 27^{o}C

5.1 Preparation of reagent bottles for cell culture.

a. Reagent bottles with closed cap are delivered non-sterile from vendor. Cap was removed and the opening was covered with aluminium foil.

b. BOLA screw-caps were placed either in sterilizing bag or suitable container.

c. Bottles and caps were sterilized by autoclaving (121^{o}C, 15 psi, 15 min).

d. After sterilization, aluminium foil was removed and replaced with BOLA-caps under laminar flow in a hood. The opening of caps was covered with AirPore tape sheet.

e. Alternatively, AirPore tape can be used directly on bottle. Using BOLA caps make it easy to take samples during culture if necessary.

f. Bottles can be sterilized with loosened BOLA caps on, covered with aluminium foil. After sterilization, aluminium foil can be replaced with AirPore tape.

5.2 Expansion of cells for expression culture

a. Sf9 or Sf21 cells adapted to suspension culture in desired medium were grown to a log phase i.e. maintaining viable count in the range of (2-5) x 10^6 cells per mL and viability greater than 95%. This serves as master stock.

b. An aliquot of cells was taken from the master stock and diluted with fresh medium to initiate 50 mL starter culture in 250mL Erlenmeyer flask. Recommended starter culture density is (0.7-0.8) x 10^6 cells per mL.

c. Diluted culture was incubated at 27^{o}C with 90 rpm in a shaker incubator (Kuhner iSF-4, 50 mm rotating diameter).

d. Cell count and viability were analyzed using Vi-Cell^{TM} XR cell counter (Beckman Coulter). It is recommended to check cell parameters every day for first few days which will give an idea on frequency of passage required. We passage cells two times a week, on day 3 and day 7.

e. Cells were passaged once the cells reach late log phase. Eg. When cells are grown in Sf900III media it is around the count of (8-9) x 10^6 cells per mL. Cells were then diluted by adding fresh medium to density of (0.7-0.8) x 10^6 cells per mL, as mentioned in point 2 above.

f. Note: if you are new to cell culture and starting your first cell culture in suspension, please follow suppliers instruction to revive cells from frozen stock.

5.3 Expression of target protein

a. Take cells maintained at early to mid-log phase. In order to have cells in best condition, dilute the culture to 1x 10^6 cells per mL a day before the initiation of expression (about 18-20 h ahead).

b. Next day adjust the cell count to around 1.8 x 10^6 cells per mL.

c. Infect the culture by adding V2 virus (4mL V2 virus per liter of culture to start with). If optimization is needed, medium scale expression can be carried out in 50-250mL volume using different ration of virus to culture.

d. Check the cells for the sign of infection if possible after 6-8h. If infection is initiated in the morning you can check late afternoon, if not next day morning. If infection is potent about 1μm increase in cell diameter can be overserved within 6-8h post infection.

e. Check the cells 24h post infection as well. If the count is double, dilute culture back to about 1.8 x 10^6 cells per mL.

f. Furthermore, check 48h post infection. Most of the time culture is ready to harvest. Parameters like cell viability and increase in cell diameter are indicators of good infection. Our preferred harvesting point is when viability is around 85-90% with significant increase in cell diameter from the time of infection.

g. Once harvesting time is identified, centrifuge the culture at 2500-3500 rpm for 10 min. Carefully decant the supernatant and discard aseptically. Collect the pellet and proceed to purification or store it at -80^{o}C until ready to purify.

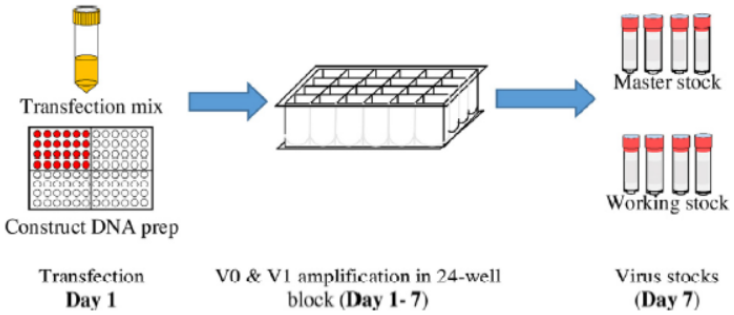
Further readings:

Please go through following article for hassle free Transfection

Roest et al. (2016) Transfection of insect cell in suspension for efficient baculovirus generation. MethodsX. 2016 May 4;3:371-7. doi: 10.1016/j.mex.2016.04.011. eCollection 2016.

Click here for full article

GRAPHICAL ABSTRACT



ABSTRACT

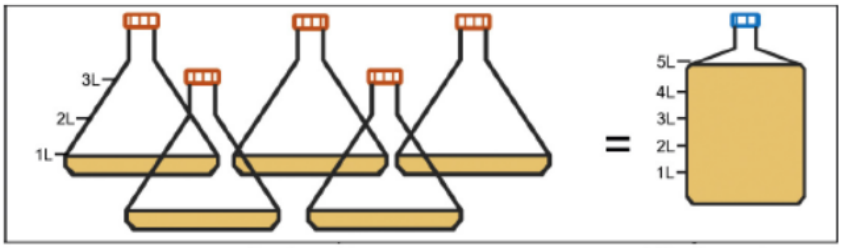
Baculovirus (BV) mediated insect cell expression system utilizes transfection as a first step to introduce recombinant baculovirus DNA into insect cells. Many labs are still relying on the conventional liposome based transfection method in adherent culture. Here we describe a more efficient method that can replace the existing method. This method is economical and does not require any special adjustment in existing labs.

- An innovative method of transfecting insect cells in suspension using polyethyleneimine (PEI) is described here.
- The beauty of this method is minimal intermediate manipulation of culture during transfection and virus generation.
- The method significantly reduces the chances of cross contamination of viruses while handling multiple targets and constructs as well as the other microbial contamination.

Another common observation in terms of protein expression for medium to large scale is a historical belief that cells need to be grown with a very high volume of air space in a culture vessel in order to have sufficient amount of air space. We have tested this extensively for large number of targets and identified an innovative economic solution. Please refer to the following article for the further details.

Insect cell culture in reagent bottle by Rieffel et al, 2014. The method paper compares the growth and maintenance of culture for protein expression in different culture vessel.

GRAPHICAL ABSTRACT



ABSTRACT

Growing insect cells with high air space in culture vessel is common from the early development of suspension cell culture. We believed and followed it with the hope that it allows sufficient air for optimal cell growth. However, we missed to identify how much air exactly cells need for its growth and multiplication. Here we present the innovative method that changed the way we run insect cell culture. The method is easy to adapt, cost-effective and useful for both academic and industrial research labs. We believe this method will revolutionize the way we run insect cell culture by increasing throughput in a cost-effective way.
In our study we identified:

- Insect cells need to be in suspension; air space in culture vessel and type of culture vessel is of less importance. Shaking condition that introduces small air bubbles and maintains it in suspension for longer time provides better oxygen transfer in liquid. For this, high-fill volume in combination with speed and shaking diameter are important.
- Commercially available insect cells are not fragile as original isolates. These cells can easily withstand higher shaking speed.

More work related to BEVS from our group is highlighted in following articles:

- Baculovirus expression vector system: an emerging host for high-throughput eukaryotic protein expression. Shrestha B, Smee C, Gileadi O. Methods Mol Biol. 2008;439:269-89. doi: 10.1007/978-1-59745-188-8_19.
- Isotope labeling of proteins in insect cells. Skora L, Shrestha B, Gossert AD. Methods Enzymol. 2015;565:245-88. doi: 10.1016/bs.mie.2015.05.013.
- Affordable uniform isotope labeling with (2)H, (13)C and (15)N in insect cells. Sitarska A, Skora L, Klopp J, Roest S, Fernández C, Shrestha B, Gossert AD. J Biomol NMR. 2015 Jun;62(2):191-7. doi: 10.1007/s10858-015-9935-6.