



Insect Cell Culture- General Information

The utilization of insect cell culture for heterologous protein expression has steadily increased over the last several decades. It has become a common expression system for both basic research and large-scale commercial applications. Currently, some of the common uses of insect cell expression include the research driven gene expression for protein crystallography-small molecule interaction, and FDA regulated GMP protein production. These regulated applications can include subunit vaccine production as well as the in vitro diagnostic and in vivo therapeutic markets. A key factor to the popularity of insect cell expression is the ability of insect cells to produce relatively large quantities of posttranslationally modified eukaryotic proteins in a relatively short period of time. Most insect cell-produced proteins have been expressed by employing the Baculovirus Expression Vector System (BEVS); however, other technologies that make stable transfected insect cells are gaining in popularity.

Many types of viruses infect insects, with the most common belonging to the family Baculoviridae. The most popular invertebrate expression vector system is based on the *Autographa Californica* nuclear polyhedrosis virus (AcNPV), an insect baculovirus isolated from the Alfalfa looper that replicates in the nucleus of over 30 lepidopteran insect cell lines. The baculovirus expression vector system has been used to express genes derived from viruses, fungi, bacteria, plants, and animals. In this system, foreign genes placed under the control of the strong polyhedrin promoter of the AcNPV are usually expressed at high levels in cultured lepidopteran insect cells. Baculovirus constitute one of the largest known groups of viruses, and they are capable of infecting over 500 species of insects, and more recently, these viruses have shown the ability to make ideal vectors for a variety of mammalian cell lines. The most widely used lepidopteran cells for BEVS are the Sf9 and Sf21 cell lines isolated from ovarian tissue of the fall army worm, *Spodoptera frugiperda*, and the High Five cell line, designated BTI-Tn-5B1-4, originally established from the *Trichoplusia ni* embryonic tissue. Sf9 cells are a sub-clone of the Sf21 cells and were selected for their faster growth rate and higher cell densities than the Sf21 cells. *Spodoptera frugiperda* cells, either Sf9 or Sf21 are preferred for virus expansion. Sf21 cells can compare favorably, in terms of heterologous protein expression, to both High Fives and the Sf9 cell lines in certain situations.

The BEVS technology, developed by Max Summers, Gale Smith and colleagues at Texas A&M University, has unique biological advantages over bacterial, yeast or mammalian protein expression systems. A major advantage is the quick turnaround time for the expression of recombinant proteins that show biological activity, antigenicity, and immunogenicity similar to authentic natural proteins. Also, the vectors are not dependent on helper viruses nor are they pathogenic for vertebrates or plants. The replication cycle is biphasic, with gene expression occurring as a cascade of sequentially and temporally regulated events. During these events two different forms of the virus are synthesized: extracellular (or budded) virus particles (ECV) for cell to cell infection within the insect and viruses contained in occlusion bodies (OV) for spread to a new host insect.



Polyhedrin is the major viral-encoded structural protein that comprises the crystalline matrix of the occlusion body that protects the virus from environmental factors that would otherwise inactivate the ECV. Upon entry into the nucleus of a susceptible cell, the virus is uncoated and transcripts of many early genes are synthesized. DNA replication begins at around 6 hours post-infection, and expression of many of the early genes is repressed as the late genes become activated. Between 10 and 48 hours post-infection, extracellular viruses bud from the plasma membrane and spread the infection. Viral occlusions are detected by 18 hours post-infection and continue to accumulate for 4 to 5 days until the infected cells lyse.

In laboratory culture, the production of OV is not necessary for survival of the virus. In vitro replication and infection does not require production of polyhedrin protein. Cell-to-cell infection in vitro takes place through ECVs. This key observation led to the development of what is referred to as BEVS for the expression of recombinant proteins. BEVS is based upon the fact that nonessential polyhedrin protein is produced in large quantities. Molecular biologists have taken advantage of this finding by replacing the polyhedrin gene with a gene of choice using conventional recombination techniques. A helper virus containing the gene of choice is cotransfected with a linearized, polyhedrin gene-deleted baculovirus. Homologous recombination between the helper virus and the baculovirus result in the gene of interest under control of the strong polyhedrin promoter, and the virus, rather than expressing polyhedrin protein, will express the heterologous recombinant protein. Foreign genes incorporated into the DNA of baculoviruses and expressed under the control of this strong viral promoter have produced abundant quantities (up to 50% of total cellular protein) of many biologically active proteins. Very late transcription from the polyhedrin promoter also allows expression of cytotoxic recombinants products long after infectious progeny are released. Two or more proteins can be expressed simultaneously in the BEVS.

Baculovirus-infected insect cells perform many the postranslational modifications of higher eukaryotes including phosphorylation, glycosylation, authentic peptide cleavage, and others, which is not the case with other vector systems such as yeast or prokaryotic systems. Recent studies of both homologous and heterologous proteins obtained from insect cell sources have revealed the presence of a limited number of hybrid and complex type N-glycan structures. Because BEVS is a lytic process it has certain limitations that can be overcome by using a stably transfected cell line.

Various culture conditions are known to influence the infection of lepidopteran cells by baculoviruses. These include temperature, pH, dissolved oxygen, osmolality and nutrient composition of the culture medium. Most lepidopteran cells proliferate best at temperatures between 25 and 28° C with an optimum pH of 6.2. The use of CO₂ is not required.



ESF 921 MEDIUM FOR INSECT CELL CULTURE

ESF 921 medium was developed to address the need for a single medium that can support high cell growth using Sf9, Sf21, Tni (e.g., High Fives and Tn 368 cells) and *Drosophila* cells (e.g., S2). ESF 921 has demonstrated high levels of recombinant protein expression using a variety of cell lines with either the BEVS technology or the stable transformed cell technology. ESF 921 was designed to especially enhance the expression of glycosylated proteins.

Cell Line	Source	Cell Density in Culture
Sf9	<i>Spodoptera frugiperda</i> ovarian tissue	>12 x 10 ⁶ cells/ml in suspension on day 6-7 with >98% viability
Sf21	<i>Spodoptera frugiperda</i> ovarian tissue	>6 x 10 ⁶ cells/ml in suspension on day 6-7 with >98% viability
Tn5B1-4 (High Fives)	<i>Trichoplusia ni</i> embryonic tissue	>8 x 10 ⁶ cells/ml in suspension on day 6-7 with >98% viability
S2	<i>Drosophila</i>	> 50-100 x 10 ⁶

GENERAL HANDLING TECHNIQUES FOR INSECT CELL CULTURE ADAPATION OF CELLS TO ESF 921 MEDIUM

Cells previously cultured in serum containing medium or serum free medium will require adaptation in ESF 921 medium to obtain the superior growth and productivity observed using ESF 921 medium. Adaptation of cells to ESF 921 medium requires several serial passages in the medium before the adaptation process is considered complete. Adaptation is an absolute requirement but will result in faster generation times, higher cell densities, and greater productivity. Non-adapted cells will not perform to potential.

Adaptation can be accomplished by serially passaging (subculture) cells in suspension culture. Initially subculture from the original medium into ESF 921 medium at a cell density of 10⁶ viable cells per ml. Allow the culture to reach 3-5 x 10⁶ viable cells per ml and subculture into fresh media at a cell density of 10⁶ viable cells per ml. Continue this until population densities reach greater than 8-10 x 10⁶ per ml on day 6-7 post subculture. When cells become adapted to ESF 921 medium, subculture stock cultures twice per week at 5 x 10⁵ viable cells per ml. Complete adaptation to ESF 921 medium may require as many as 10-20 passages in ESF 921 medium depending on prior culture medium. These cell densities are based upon the adaptation of Sf9 cells. Sf21 and Tni cells should be passed upon reaching 5 x 10⁶ cells per ml. S2 cells should reach greater than 25 x 10⁶ cells per ml before passaging.



It is generally recommended that cell culture medium should be equilibrated to room temperature prior to use. However, experience has shown that media can be used directly from the refrigerator without adverse effect.

MONOLAYER CULTURE

1. Aspirate and discard the medium and floating cells from a confluent monolayer (a monolayer will reach confluency in 4 to 6 days). When removing medium from monolayer cultures in tissue-culture flasks, tilt the flasks at an angle toward bottom corner of the flask. Allow media to flow to this corner and carefully remove the media with a pipette.
2. Add 5 ml of fresh ESF 921 medium to the monolayer in a 25 cm² tissue culture flask (or 15 ml to a 75 cm² flask).
3. Suspend cells by rapping the flask against the palm of your hand and/ or vigorously pipetting the medium across the monolayer with a sterile pipette.
4. Observe the cell monolayer using an inverted microscope to insure complete cell detachment from the surface of the flask.
5. Perform a viable cell count on harvested cells using trypan blue exclusion.
6. Inoculate a new 25 cm² flask with 1.0-1.25 x 10⁶ cells in 5 ml of complete medium (3.0-3.75 x 10⁶ cells in 15 ml of medium for a 75 cm² flask).
7. Return cultures to incubator (27° C \pm 0.5 C). Loosen caps. Incubate for 4 to 6 days. Note: for slower-growing cultures, it may be necessary to feed them on day 3 post-planting. Aspirate the spent medium without disrupting the monolayer, and gently add fresh medium

SHAKER CULTURE

1. Inoculate a 250 ml Erlenmeyer flask with 100 ml of complete medium containing 5 x 10⁵ viable cells per ml (use 50 ml of medium for a 125 ml flask).
2. Set the orbital shaker at 120 – 150 rpm, sometimes even more if oxygen is in great demand for your particular culture. Cells are properly protected from shear stress by ESF921 formulation. Note: The orbital shaker/flask assembly should be maintained at 27 ° C in a non-humidified, non-gas regulated environment. Loosen caps so that there is plenty of free play (¼ inch when pulling cap in an up and down motion). This will facilitate oxygen transfer allowing cells to proliferate at a maximum rate.
3. Subculture cells to 5 x 10⁵ cells per ml twice weekly, i.e., Monday-Thursday routine. Note: Inoculating cells at too low of a density may result in prolong lag times and thus slower doubling times.
4. Cultures may be gently centrifuged as needed at 1000 rpm for 5 minutes, and pellets may be resuspended in fresh medium to reduce accumulation of cell debris and toxic by products. Note: Centrifuging cells more than once a month is not recommended. A healthy culture should not require this procedure and a new stock of cells should be initiated.



SPINNER CULTURE

1. Determine viable cell count of cell suspension to be used for inoculating spinner flask.
2. Dilute cell suspension to 5×10^5 viable cells per ml in ESF 921 medium.
3. Inoculate spinner flask with enough cell suspension to at least reach mid-paddle level due to agitation requirements. Maximum fluid level is variable depending on vessel size, but should not exceed $\frac{1}{2}$ of the vessel capacity due to oxygenation requirements.
4. Loosen the side-arm caps to facilitate oxygen transfer. Note: Make sure caps are loose enough to facilitate maximal oxygen transfer. Non-viable cultures that have become white, without the pungent smell of bacteria, is usually a sign that the cells suffocated because of poor oxygen exchange.
5. Incubate spinner flask at 27°C at a constant stirring rate of 125-150 rpm.
6. Subculture new spinner cultures twice weekly at a density of 5×10^5 per ml.

INFECTION OF INSECT CELLS FOR RECOMBINANT PROTEIN EXPRESSION

The most important consideration for robust recombinant protein expression is the health of the cell culture. It is essential that the cells are in log phase at the time of infection. The stock cells used for the experiment should come from a culture in mid-exponential growth phase. Growing cells up to a high density to increase the number of cells in a small volume is not advised. It is preferable to use a larger volume of cells at a lower density.

1. Seed the experimental culture at between $0.5-1 \times 10^6$ cells per ml and allow to grow overnight.
2. Confirm log growth of cells by observation of increase cell counts. If the culture did not grow overnight, discard and start again.
3. Infect the culture at your desired MOI

$$\text{Volume of inoculum needed} = \frac{\text{MOI (iu/cell)} \times \text{number of cells}}{\text{Titer of virus (iu/ml)}}$$

The MOI used should be determined by small scale optimization prior to a large scale expression. Using a low MOI can boost expression by allowing for cell growth and utilizing secondary infection, resulting in a greater final infection density. Alternatively an MOI of 3 is often used to infect all cells at once. Poor culturing conditions can lead to inconsistent results as lag phase cells will not become productively infected with virus.

4. Harvest at desired time of harvest (TOH).
 - a. TOH is dependent upon many factors including stability of product, sensitivity to proteases, secreted versus intracellular versus membrane bound. It is advisable to perform a TOH timecourse prior to large scale production



Culture parameters for expression of recombinant proteins using the Baculovirus Expression Vector System can be highly protein specific. Expression Systems Technical Support is available to help with your particular protein.