Invitrogen™
life technologies

Instruction Manual

Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques

INSIDE FRONT COVER

Table of Contents

Tables:

1. Introduction

Recombinant baculoviruses are widely used to express heterologous genes in cultured insect cells and insect larvae. For large-scale applications, the baculovirus expression vector system (BEVS) is particularly advantageous. Specialized media, transfection reagents, and vectors have been developed in response to recent advances in insect cell culture and molecular biology methods.

The following are important choices in designing a system for recombinant protein production:

- Selecting the expression vector, including the style or type of promoter, that provides best results with the recombinant gene product being expressed.
- Evaluating insect cell lines, growth media (serum-supplemented or serum-free), and feeding/infection strategies that allow for optimal rAcNPV and/or product expression.
- Choosing a scalable process of cell culture and deciding on other factors affecting downstream processing.

Overview of Baculovirology

Baculoviruses are the most prominent viruses known to affect the insect population. They are double-stranded, circular, supercoiled DNA molecules in a rod-shaped capsid (1). More than 500 baculovirus isolates (based on hosts of origin) have been identified, most of which originated in arthropods, particularly insects of the order Lepidoptera (2,3). Two of the most common isolates used in foreign gene expression are *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV).

Wild-type baculoviruses exhibit both lytic and occluded life cycles that develop independently throughout the three phases of virus replication. The following are characteristics of the three phases:

1. ***Early Phase:*** In this phase (also known as the virus synthesis phase), the virus prepares the infected cell for viral DNA replication. Steps include attachment, penetration, uncoating, early viral gene expression, and shut off of host gene expression. Actual initial viral synthesis occurs 0.5 to 6 h after infection.
2. ***Late Phase:*** In this phase (also known as the viral structural phase), late genes that code for replication of viral DNA and assembly of virus are expressed. Between 6 and 12 h after infection, the cell starts to produce extracellular virus (EV), also called non-occluded virus (NOV) or budded virus (BV). The EV contains the plasma membrane envelope and glycoprotein (gp)64 necessary for virus entry by endocytosis. Peak release of extracellular virus occurs 18 to 36 h after infection.
3. ***Very Late Phase:*** In this phase (also known as the viral occlusion protein phase), the polyhedrin and p10 genes are expressed, occluded virus (OV)—also called occlusion bodies (OB) or polyhedral inclusion bodies (PIBs)—are formed, and cell lysis begins. Between 24 and 96 h after infection, the cell starts to produce OV, which contains nuclear membrane envelopes and the viral polypeptides gp41 and gp74. Multiple virions are produced and surrounded by a crystalline polyhedra matrix. The virus particles produced in the nucleus are embedded within the polyhedrin gene product and a carbohydrate-rich calyx.

Infection

Figure 1 summarizes how baculoviruses infect cells and are transmitted *in vivo* vertically and horizontally. During the lytic cycle, enveloped and budded virions are generated. These virions promote horizontal transmission of the infection throughout the tissue in an *in vivo* infection of a worm larvae, or throughout the cell culture in an *in vitro* overexpression system. *In vitro,* this cycle is exploited to both generate virus stocks and establish a fully developed infection from subsaturating primary virus inocula. During the occluded cycle, virions packaged in the PIBs are generated. *In vivo*, these virions promote vertical transmission of the virus from insect host to insect host. *In vitro,* a polyhedrin gene modified to express a recombinant gene product in place of the PIBs is used. Biochemically, the essential difference between the lytic and occluded cycles is the induction of polyhedrin production at the beginning of the very late phase.

You need to be able to distinguish between the initiation of virus production and budding, at approximately 8 to 10 h post-infection, and the initiation of protein expression under control of the polyhedrin promoter, at approximately 20 to 24 h. By doing so, you will be able to effciently produce high-titer baculovirus stocks and high-quality recombinant product (*i.e.,* product that is non-degraded and free of cell debris).

Vertical Transmission

After the OV is ingested by insect larvae, the crystalline polyhedrin matrix is degraded in the alkaline mid-gut of the insect. Embedded virions are released and fuse to microvillar epithelial cells. Infected cells release EV from the basement membrane side of the mid-gut cell into the hemolymph system.

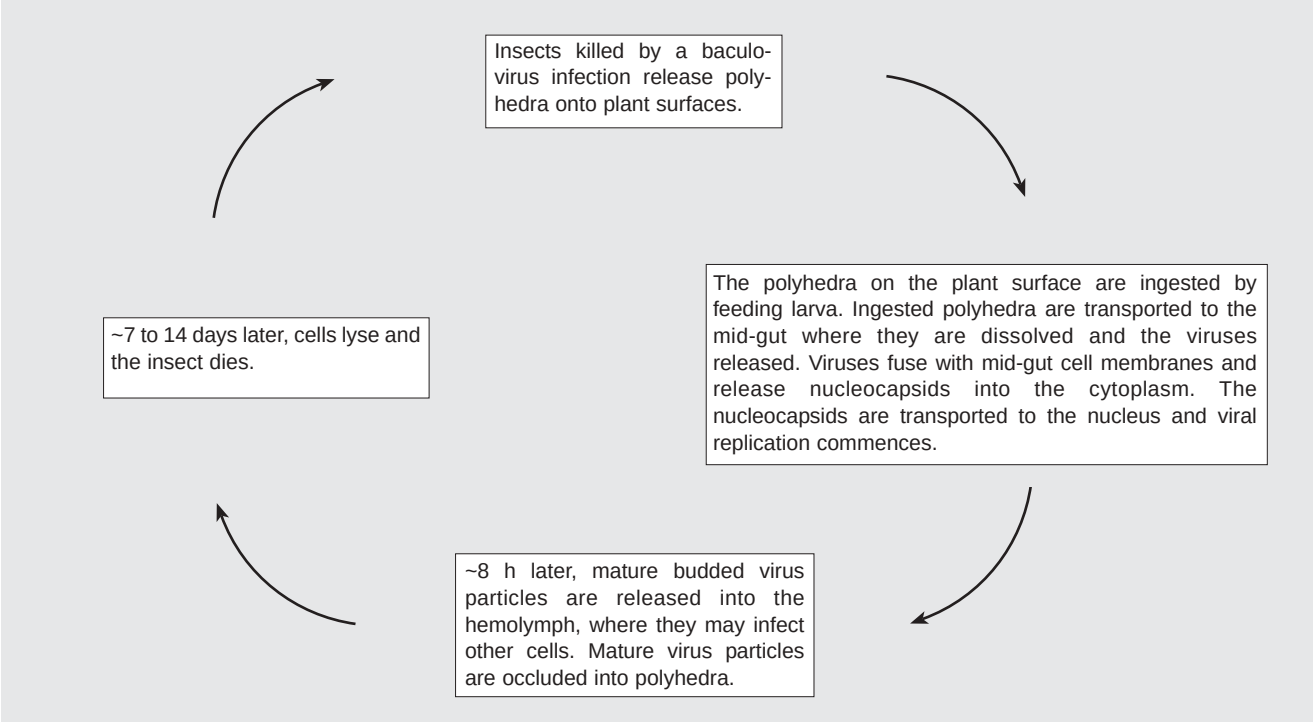
Horizontal Transmission

EV enters the insect hemocoel and immediately spreads throughout the insect's open circulatory system, infecting many cell types. Within 10 viral generations, the insect dies and the OV, produced during the very late stage of infection, is released into the environment.

Baculoviruses as Expression Vectors

The major difference between the naturally occurring *in vivo* infection and the recombinant *in vitro* infection is that the naturally occurring polyhedrin gene within the wild-type baculovirus genome is replaced with a recombinant gene or cDNA. These genes are commonly under the control of polyhedrin and p10 promoters. In the late phase of infection, the virions are assembled and budded recombinant virions are released. However, during the very late phase of infection, the inserted heterologous genes are placed under the transcriptional control of the strong AcNPV polyhedrin promoter. Thus, recombinant product is expressed in place

FIGURE 1. *In vivo* baculovirus infection and replication.



of the naturally occurring polyhedrin protein. Usually, the recombinant proteins are processed, modified, and targeted to the appropriate cellular locations.

Cytopathogenesis

As the recombinant infection advances, several morphological changes take place within the cells. The timing of the infection cycle and the changes in cell morphology vary with the insect cell line and strain of baculovirus used. The metabolic condition of the culture and growth medium used also can affect the timing of baculovirus infection. The following morphological changes are typical of monolayer Sf9 cells infected with recombinant AcNPV.

1. ***Early Phase:*** Infection begins with the adsorptive endocytosis of one or more competent virions by a cell in a high metabolic state (peak replication rate). The nucleocapsids pass through the cytoplasm to the nucleus. When the virions enter the nucleus, they release the contents of the capsid. Within 30 min of infection, viral RNA is detectable. Within the first 6 h of infection, the cellular structure changes, normal cellular functions decline precipitously, and early-phase proteins become evident.
2. ***Late Phase:*** Within 6 to 24 h after infection, an infected cell ceases many normal functions, stops dividing, and is logarithmically increasing production of viral genome and budded virus. The virogenic stroma (an electron-dense nuclear structure) becomes well developed. Infected cells increase in diameter and have enlarged nuclei. The cells may demonstrate reduced refractivity under phase contrast microscopy. Infected cultures stop growing.
3. ***Very Late Phase:*** Within 20 to 36 h after infection, cells cease production of budded virus and begin the assembly, production, and expression of recombinant gene product. In monolayer cultures, areas of infection display decreased density as cells die and lyse. Likewise, in suspension cultures, cell densities begin to decrease. Infected cells continue to be increased in diameter and have enlarged nuclei. The cytoplasm may contain vacuoles, and the nuclei may demonstrate granularity.

   As the infected cells die, plaques develop in immobilized cultures. The plaques can be identified under a microscope as regions of decreased cell density, or by eye as regions of differential refractivity.

Advantages of BEVS Technology

Since 1983, when BEVS technology was introduced, the baculovirus system has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression (4). More than 600 recombinant genes have been expressed in baculoviruses to date. Since 1985, when the first protein (IL-2) was produced in large scale from a recombinant baculovirus, use of BEVS has increased dramatically (5). Baculoviruses offer the following advantages over other expression vector systems.

- ***Safety:*** Baculoviruses are essentially nonpathogenic to mammals and plants (6). They have a restricted host range, which often is limited to specific invertebrate species. Because the insect cell lines are not transformed by pathogenic or infectious viruses, they can be cared for under minimal containment conditions. Helper cell lines or helper viruses are not required because the baculovirus genome contains all the genetic information.
- ***Ease of Scale Up:*** Baculoviruses have been reproducibly scaled up for the large-scale production of biologically active recombinant products.

- ***High Levels of Recombinant Gene Expression:*** In many cases, the recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished.
- ***Accuracy:*** Baculoviruses can be propagated in insect hosts which post-translationally modify peptides in a manner similar to that of mammalian cells.
- ***Use of Cell Lines Ideal for Suspension Culture:*** AcNPV is usually propagated in cell lines derived from the fall armyworm *Spodoptera frugiperda* or from the cabbage looper *Trichoplusia ni*. Cell lines are available that grow well in suspension cultures, allowing the production of recombinant proteins in large-scale bioreactors.

Generating a Recombinant Virus by Homologous Recombination

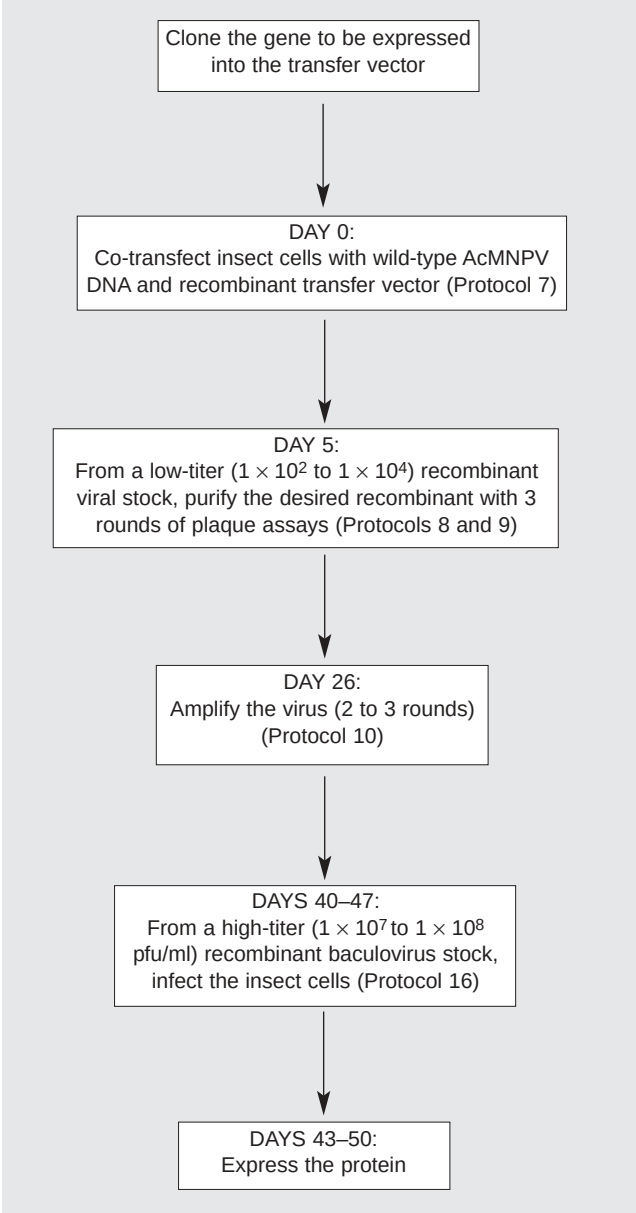
Using homologous recombination to generate a recombinant baculovirus is outlined in figure 2. The most common baculovirus used for gene expression is AcMNPV. AcMNPV has a large (130-kb), circular, double-stranded DNA genome. The gene of interest is cloned into a transfer vector containing a baculovirus promoter flanked by baculovirus DNA derived from a nonessential locus—in this case, the polyhedrin gene. The gene of interest is inserted into the genome of the parent virus (such as AcMNPV) by homologous recombination after transfection into insect cells. Typically, 0.1% to 1% of the resulting progeny are recombinant. The recombinants are identified by altered plaque morphology. For a vector with the polyhedrin promoter, as in this example, the cells in which the nuclei do not contain occluded virus, contain recombinant DNA. Detection of the desired occlusion-minus plaque phenotype against the background of greater than 99% wild-type parental viruses is difficult.

A higher percentage of recombinant progeny virus (nearly 30% higher) results when the parent virus is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome (7,8). To obtain an even higher proportion of recombinants (80% or more), linearized viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene can be used (9). These approaches can take more than a month to purify plaques, amplify the virus, and confirm the desired recombinants.

Generating a Recombinant Virus by Site-Specific Transposition

A faster approach for generating a recombinant baculovirus (10,11) uses site-specific transposition with Tn7 to insert foreign genes into bacmid DNA propagated in *E. coli*. The gene of interest is cloned into a pFastBac™ vector, and the recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotic selection and blue/white screening, since the transposition results in disruption of the *lac*Zα gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells. The steps to generate a recombinant baculovirus by site-specific transposition using the Bac-to-Bac™ Baculovirus Expression System are outlined in figure 3.

FIGURE 2. Generating a recombinant baculovirus by homologous recombination.



A variety of pFastBac donor plasmids are available which share common features. The plasmid pFastBac 1 (11) is used to generate viruses which will express unfused recombinant proteins. The pFastBac HT series of vectors (12) are used to express polyhistidine-tagged proteins which can be rapidly purified on metal affinity resins. The pFastBac DUAL vector has two promoters and cloning sites, allowing expression of two genes, one from the polyhedrin promoter and one from the p10 promoter.

Advantages of Site-Specific Transposition: Using site-specific transposition has two major advantages over homologous recombination:

- ***One-Step Purification and Amplification:*** Because recombinant virus DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus, multiple rounds of plaque purification are not required and identification of the recombinant virus is easier. In 7 to 10 days, you will have pure recombinant virus titers of $>1 \times 10^7$ pfu/ml without any viral amplification.
- ***Rapid and Simultaneous Isolation of Multiple Recombinant Viruses:*** This feature is particularly valuable for expressing protein variants in structure/function studies.

Insect Cell Culture Techniques

Successful culture of insect cells requires a basic familiarity with insect cell physiology and general cell culture methods. The materials and methods for use with insect cell culture have evolved and contributed to the advancement of BEVS technology. The following factors have been significant:

- Growth supplements and shear force protectants are widely used.
- Serum-free media (SFM) have replaced serum-supplemented media, particularly for large-scale production.
- Some insect cell lines have been optimized for use in suspension culture, especially useful for scale-up.

Cell Lines

The most common cell lines used for BEVS applications are listed in table 1. Of these, Sf9, a clonal isolate of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE, is probably the most widely used. Sf9 was originally established from ovarian tissue of the fall armyworm (13). Although there is significant scientific data on the characteristics of this Lepidopteran cell line, it remains to be confirmed whether it is the best line for virus or recombinant protein production. Ongoing research suggests that different insect cell lines may support varying levels of expression and differential glycosylation with the same recombinant protein (14).

TABLE 1. Insect cell lines commonly used in BEVS applications.

Insect Species	Cell Line
Spodoptera frugiperda	Sf9
Spodoptera frugiperda	Sf-21
Trichoplusia ni	Tn-368
Trichoplusia ni	High-Five™ BTI-TN-5B1-4

Note: Each of these cell lines has been successfully adapted to suspension cultures.

Media and Growth Supplements

Commonly used insect cell culture media are listed in table 2. Traditionally, Grace's Supplemented (TNM-FH) medium has been the medium of choice for insect cell culture. However, other serum/hemolymph-dependent and serum-free formulations have evolved since Grace's medium was introduced.

FIGURE 3. Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC expression system.

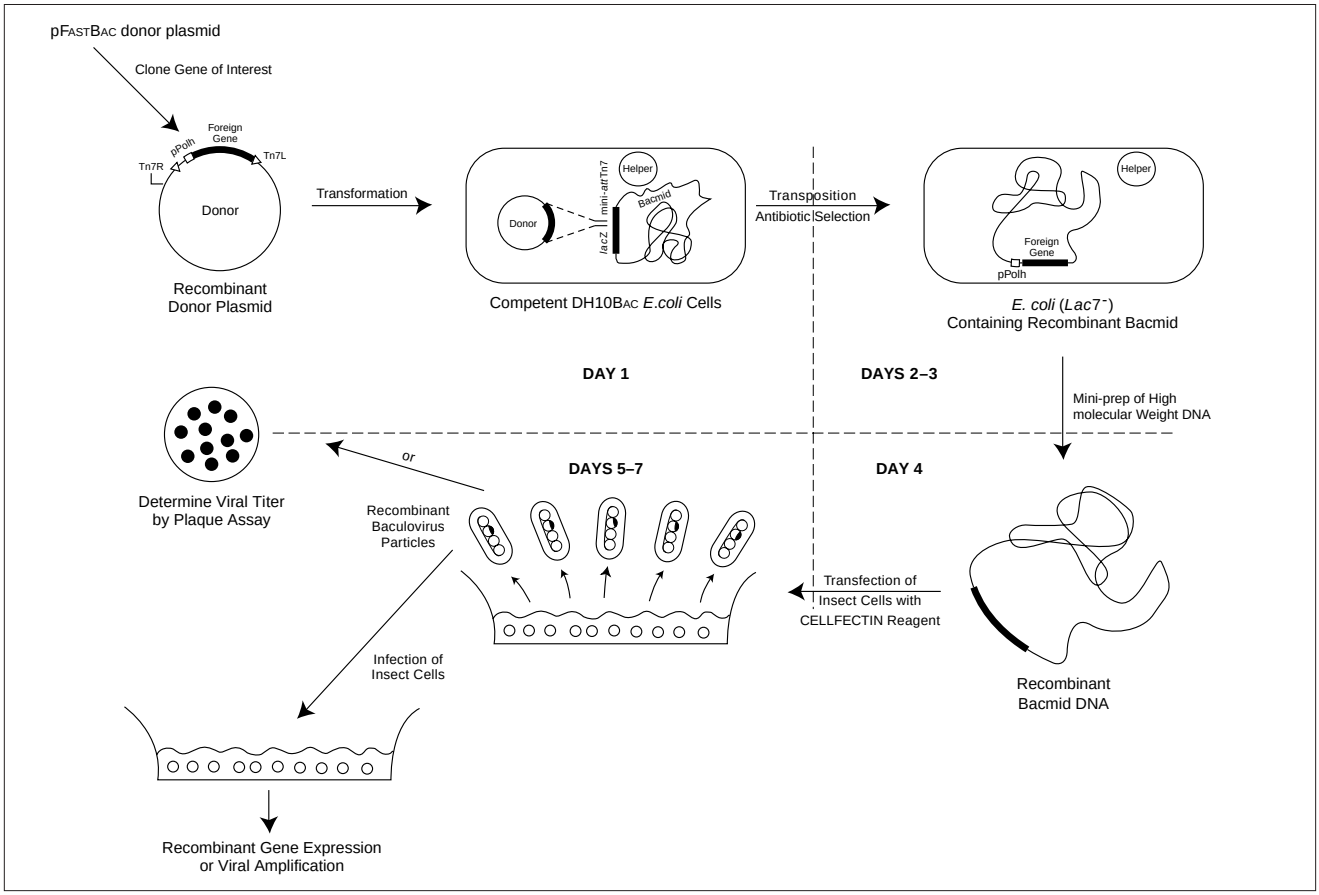


TABLE 2. Insect cell culture media commonly used in BEVS applications.

Serum/hemolymph-dependent media	Serum-free media
Grace's Supplemented (TNM-FH)	Sf-900 II SFM
IPL-41	EXPRESS-FIVE™ SFM
TC-100	
Schneider's Drosophila	

Note: Store liquid media which all contain photolabile components in the dark at 4°C to 8°C.

Fetal bovine serum (FBS) has been the primary growth supplement used in insect cell culture medium. FBS has almost completely supplanted the first major supplement, insect hemolymph, which tended to melanize and deteriorate the quality of the culture medium (15). Of the more than 100 insect cell culture media described in the literature, a majority contain, or recommend, varying concentrations of serum as a growth supplement (16).

Supplementation with serum has both desirable and undesirable effects. These are summarized in table 3. Serum and other undefined supplements, such as lactalbumin hydrolysate and yeastolate, provide cells with growth-promoting factors such as amino acids, peptides, and vitamins, which may not be available in defined, basal media formulations.

TABLE 3. Effects of serum on cell cultures.

Desirable Effects	Undesirable Effects
Promotes growth	May cause excessive foaming in sparged bioreactors
Provides shear force protection	May introduce adventitious agents
Protects against proteolytic degradation and environmental toxicities	Increases cost and complexity of downstream processing
	Fluctuates in price, quality, and availability
Contributes cellular attachment factors	May demonstrate suboptimal cell growth or toxicity
	May demonstrate decreased product yields

Before 1984, few scientific articles referenced serum-free insect cell culture media. At that time, serum-free insect culture media were used mostly to replicate insect viruses for production of viral pesticides. These early SFM formulations were not well suited for use in producing recombinant proteins. Early formulations contained inherent flaws that limited cellular growth, suspension culture, and protein expression. For BEVS applications, these early formulations were generally poorly defined and too rich in protein.

Most commercially available serum-free insect media are essentially simple variations of IPL-41 basal medium supplemented with undefined protein hydrolysates and a lipid/surfactant emulsion (17). Second-generation serum-free formulations such as Sf-900 II SFM and EXPRESS-FIVE SFM are specifically designed for large-scale production of recombinant proteins. They contain optimized concentrations of amino acids, carbohydrates, vitamins, and lipids that reduce or eliminate the effect of rate-limiting nutritional restrictions or deficiencies. Both Sf-900 II SFM and EXPRESS-FIVE SFM support faster population doubling times and higher saturation cell densities than do traditional media. Thus, you can obtain both higher wild-type or recombinant baculovirus titers and increased levels or yields of recombinant protein expression by using these formulations. The optimized formulations offer the following advantages over sera:

- Eliminate the need for costly fetal bovine and other animal sera supplements
- Increase cell and product yields
- Eliminate adventitious agents
- Have lot-to-lot consistency

Environmental Factors

Invertebrate cell cultures are extremely sensitive to environmental factors and conditions. The low-protein nature of most serum-free formulations often increases cellular sensitivity. To reduce problems, use materials and equipment designated for tissue culture use only, including incubators, flow hoods, autoclaves, media preparation areas, specialty gases, and bio-reactors. Follow the guidelines listed here to ensure that the physical conditions of your culture optimize growth.

Temperature: The optimal range for growth and infection of most cultured insect cells is 25°C to 30°C. Healthy serum-supplemented monolayer cultures can be stored at 2°C to 8°C for periods up to 3 months.

pH: The pH of a growth medium affects both cellular proliferation and viral or recombinant protein production. Although many values have been reported for invertebrate cells, in most applications a pH range of 6.0 to 6.4 works well for most lepidopteran cell lines. The insect media described in this guide will maintain a pH in this range under conditions of non-CO_2 equilibration and open-capped culture systems.

Osmolality: The optimal osmolality of medium for use with lepidopteran cell lines is 345 to 380 mOsm/kg. To maintain reliable and consistent cellular growth patterns and minimize technical problems, maintain pH and osmolality within the ranges listed here.

Aeration: Invertebrate cells require sufficient transfer of dissolved oxygen by either passive or active methods for optimal cell proliferation and expression of recombinant proteins. Larger bioreactor systems using active or controlled oxygenation systems require dissolved oxygen at 10% to 50% of air saturation.

Shear Forces: Suspension culture techniques generate mechanical shear forces. Factors that contribute to the total shear stresses experienced by cells in suspension culture include the size and type of impellers within stirred vessels, the size and velocity of bubbles in airlift or sparged bio-reactors, and the resulting turbulent action at the culture surface. During suspension cell culture, most insect cell lines require shear force protection. Although serum concentrations between 5% and 20% in medium appear to provide some protection from shear forces, we recommend that all suspension cultures, whether serum-free or serum-supplemented, be supplemented with a shear force protectant such as PLURONIC® F-68. (If not already present in the formulation.)

2. Protocols for Culturing Host Cells

General Materials and Equipment List

The following materials and equipment are required to culture insect cells. Additional, protocol-specific materials are listed with each protocol.

- cell line(s) negative for the presence of mycoplasma or other adventitious contaminating agents (18,19)
- electronic cell counter
- hemocytometer chamber
- incubator capable of maintaining 27°C ± 0.5°C and large enough to contain the desired culture configuration apparatus
- inverted and phase contrast light microscopes
- laminar flow hood suitable for cell culture
- low-speed centrifuge
- pipet aide, automated or manual
- pipets: 1-, 2-, 5-, 10- and 25-ml volumes
- 37°C water bath
- trypan blue
- complete serum-supplemented or serum-free medium of choice

Protocol 1: Subculturing Monolayer Cultures

Note: To ensure adequate oxygenation, maintain minimal media depth and loose caps.

Materials List

- Cell culture "T"-flasks, 25- and/or 75-cm^2

1. Aspirate and discard the medium and floating cells from an 80% to 90% confluent monolayer.
2. To each 25-cm^2 flask, add 4 to 6 ml of complete growth medium equilibrated to room temperature. If you are using 75-cm^2 flasks, add 15 ml per flask.
3. Resuspend cells by pipetting the medium across the monolayer with a Pasteur pipette.
4. Observe the cell monolayer using an inverted microscope to ensure adequate cell detachment from the surface of the flask.
5. Determine the viable cell count of harvested cells (e.g., using a hemocytometer and trypan blue dye exclusion).
6. Inoculate cells at 2×10^4 to 5×10^4 viable cells/cm^2 into 25- or 75-cm^2 flasks.
7. Incubate cultures at 27°C ± 0.5°C with loose caps to allow gas exchange.
8. Subculture the flasks when the monolayer reaches 80% to 100% confluency, approximately 2 to 4 days post-planting. The length of time needed to reach confluency before subculturing often depends on the cell inocula concentration used in step 6.

   Note: If the cell line is growing slowly, feed the flasks on day 3 or 4 post-planting. Aspirate spent medium from one side of the monolayer and gently re-feed with fresh medium. Subculture when monolayer reaches 80% to 100% confluency.

Protocol Notes

- ***Master Cell Seed Stock:*** As soon as the culture is fully adapted to the culture conditions and growth medium, prepare and cryopreserve a master cell seed stock (see Protocol 5). As some cell lines may be passage-number dependent, we recommend establishing fresh cultures periodically (e.g., every 3 months or 30 passages) from the frozen master cell seed stock.

  For Serum-Supplemented Cultures:
- ***Antibiotic Concentrations:*** 0.25 µg/ml of amphotericin B, 100 U/ml of penicillin, and 100 µg/ml of streptomycin.
- ***Care in Handling:*** Use care when moving serum-supplemented cultures of Sf9 cells. These cultures do not adhere tightly to most glass or plastic substrates.

  For Serum-Free Cultures:
- ***Antibiotic Concentrations:*** Antibiotics or antimycotics are not recommended for serum-free cultures. If you use antibiotics in serum-free culture, reduce the standard concentrations ~50%.
- ***Dislodging the Cells:*** Insect cells attach very tightly to substrates under serum-free conditions and require additional effort to detach. To dislodge the cells, you may need to shake the flask vigorously two to three times using a wrist-snapping motion. **Caution:** To avoid contamination, always tighten the cap before shaking the flask.

Protocol 2: Adapting Monolayer Cells to Suspension Culture

Because insect cells are not generally anchorage dependent, they adapt easily to suspension culture conditions. The insect cell lines commonly used in BEVS applications have all been successfully adapted to suspension cell cultures (see Appendix A). It is important to proceed slowly when adapting stationary cultures to suspension culture. You may observe a drop in viability and increased clumping through the first three to five passages. This protocol will optimize the adaptation of most invertebrate cell lines to suspension culture and reduce or eliminate cell clumping over a short period of time. Six to 10 confluent 75-cm^2 monolayer flasks are sufficient to initiate a 100-ml suspension culture.

1. Dislodge cells from the bottom of the flasks (see Protocol 1).
2. Pool the cell suspension, and determine the viable cell count.
3. Dilute the cell suspension to approximately 5×10^5 viable cells/ml in complete serum-supplemented or serum-free growth medium equilibrated to room temperature.
4. Incubate at 2.0°C ± 0.5°C with a stirring rate of 100 rpm for shaker flasks or a stirring rate of 75 rpm for spinner cultures.
5. Subculture the cells when the viable cell count reaches 1×10^6 to 2×10^6 cells/ml (3 to 7 days post-planting). Increase the stirring speed by 5 to 10 rpm with each

subsequent passage. If cell viabilities drop below 75%, decrease stirring speed by 5 rpm for one passage until culture viability recovers and is >80%.

6. For shaker flask cultures, repeat step 5 until the constant stirring speed reaches 130 to 150 rpm.

   For spinner cultures, repeat step 5 until the constant stirring speed is 90 to 100 rpm—unless the spinner flask is equipped with a micro-carrier stirring assembly (flat blade impeller), in which case limit maximum stirring speed to 75 to 80 rpm.

7. When cells have fully adapted to suspension culture, follow Protocol 3 for routine maintenance.

Protocol Notes

- ***Clumping:*** High-Five BTI-TN-5B1-4 and Tn-368 cell lines often demonstrate a severe clumping problem in serum-free suspension cultures. To minimize clumping, let the culture sit 2 to 3 min before subculturing, until the larger clumps (>10 cells per clump) settle to the bottom of the flask. Pull samples for counting and seeding new cultures from the upper third of the suspension culture (this technique selects for a cell population that grows as single cells). If necessary, repeat this step two to three consecutive passages until clumping is reduced. Even with several repetitions, 5% to 20% of the cell population may remain composed of small clumps 5 to 10 cells in size.
- ***Master Cell Seed Stock:*** As soon as the culture is fully adapted to the culture conditions and growth medium, prepare and cryopreserve a master cell seed stock (see Protocol 5). As some cell lines may be passage-number dependent, we recommend establishing fresh cultures periodically (e.g., every 3 months or 30 passages) from the frozen master cell seed stock.
- ***Surfactants:*** Do not supplement serum-free insect media with additional surfactant, such as PLURONIC F-68. Surfactants are used in serum-supplemented cultures to lessen cellular damage due to shear forces, but concentrations >0.10% may decrease growth or result in cellular toxicity in serum-free cultures. Unless otherwise indicated, most SFM contain sufficient surfactant(s) to protect cells.
- Magnetic stir bars designed to operate on the bottom of the flasks are not suitable for insect cell culture.

Protocol 3: Maintaining Suspension Cultures

The standard flasks used in a suspension culture are 250-ml disposable, sterile Erlenmeyer flasks (for volumes of 50 to 125 ml) and 250-ml glass spinner flasks (for volumes of 150 to 175 ml). Although you can scale up shaker or spinner flask cultures to a variety of vessels and volumes, you must optimize the relative flask fill volumes and stirring speeds for each configuration. See table 4 for typical medium volumes. If you use glass shake or spinner flasks, be sure the flasks are thoroughly cleaned after each use.

This protocol can be used with 250-ml shake flasks or spinner flasks, with loosened caps. The total amount of media per cell suspension volume is 50 to 125 ml for shake flasks or 175 ml for spinner flasks. Under these conditions, oxygen tensions are not rate limiting and cultures achieve maximum population doubling times and densities.

TABLE 4. Useful medium volumes.

Flask size (ml)	Shaker flask culture volume (ml)	Spinner flask culture volume (ml)
125	25–50	50–100
250	50–125	150–200
500	125–200	200–300
1,000	200–400	300–1,000
3,000	400–800	2,000–3,000

Materials List

- disposable Erlenmeyer flasks, 125-, 250-, and 500-ml
- glass spinner flasks, 125- and 250-ml
- orbital shaker fitted for 50- to 500-ml Erlenmeyer flasks, with shaking speed of up to 150 rpm
- stirring platform capable of constant operation at 90 to 100 rpm
- PLURONIC F-68, 10% (100X)

1. Maintain the orbital shaker or stirring platform in a 27°C ± 0.5°C, nonhumidified, non-CO_2 equilibrated, ambient-air regulated incubator or warm room. For cultures already adapted to and maintained in suspension culture, set orbital shaker at 135 to 150 rpm and spinner platforms at 90 to 100 rpm.
2. Remove a 1- to 2-ml sample from a 3- to 4-day-old suspension culture (in mid- exponential growth) and determine the viable cell count.
3. Dilute the cell suspension to 3×10^5 viable cells/ml in complete serum-free or serum-supplemented growth medium equilibrated to room temperature.

- *For serum-supplemented cultures:* You may add 10 ml/L PLURONIC F-68 (0.05% to 0.1% final concentration) to lessen cellular damage by shear forces.
- *For shaker flasks:* Maintain stock cultures as a 50- to 100-ml culture in 250-ml Erlenmeyer flasks.
- *For spinner flasks:* Maintain stock cultures as 150- to 175-ml cultures in 250-ml spinner flasks.

  For typical culture volumes, see table 4. To aerate the cultures, loosen the caps about ¼ to ½ of a turn.

4. Incubate cultures until they reach 2×10^6 to 3×10^6 viable cells/ml. To maintain consistent and optimal cell growth, subculture suspension cultures twice weekly.
5. Once every 3 weeks, gently centrifuge the cell suspension at 100 × g for 5 min. Resuspend the cell pellet in fresh medium to reduce the accumulation of cell debris and metabolic byproducts.

Protocol Notes

For Spinner Cultures:

- *Scalability:* The physical constraint of providing adequate oxygen tensions to the culture limits the culture's scalability. Keep the volume in the spinner vessel below 2/3 full and provide for gas sparging as the vessel size increases above 500 ml.
- *Calibration and Assembly:* Recalibrate the gradation marks on commercial spinner flasks using a graduated cylinder. Ensure the impeller mechanisms rotate freely and do not contact vessel wall or base.

- *Siliconization:* Coating cultureware with a nontoxic siliconizing agent may minimize attachment of cell debris and clumps at the media meniscus. Recoat siliconized cultureware after three to four uses. Follow the manufacturer's guidelines, and test the efficacy of siliconization for your protocols.

For Serum-Free Cultures:

- *Dilutions:* For Sf9 and Sf21 cells in SFM, do not dilute the suspension cultures below 3×10^5 cells/ml. Doing so will cause an extended growth lag of 2 to 7 days. For *Trichoplusia ni* (Tn-368 or BTI-TN-5B1-4) cells, seeding 2×10^5 cells/ml is sufficient.

Protocol 4: Adapting Cultures to Serum-Free Medium

Adapt cell cultures to SFM simultaneously through both direct and sequential adaptation. Doing so may save you valuable time if one of the methods does not work. Before adapting monolayer cells to SFM, first establish them to suspension culture (see Protocol 2). Cells must be in mid-exponential growth with a viability of at least 90%.

When the cells are completely adapted to serum-free culture, they should reach maximum densities and have population doubling times comparable to growth in serum-supplemented medium.

Materials List

- Sf-900 II SFM or EXPRESS-FIVE SFM
- Insect cells adapted to suspension culture and growth in serum-supplemented medium

Direct Adaptation to SFM:

The chief advantage to this method is time. Insect cultures can be adapted to SFM in 5 to 8 passages (~3 weeks). If viabilities decrease to <50%, or if cultures are growing slowly (population doubling times are >72 h) for more than 3 to 4 consecutive passages, use the sequential adaptation method.

1. Prewarm SFM to 27°C ± 0.5°C.
2. Transfer cells growing in medium containing 5% to 10% FBS directly into the prewarmed SFM at a density of 5×10^5 cells/ml.
3. When the cell density reaches 2×10^6 to 3×10^6 cells/ml (4 to 7 days post-seeding), subculture the cells to a density of 5×10^5 cells/ml.
4. Subculture stock cultures of SFM-adapted cells 1 to 2 times per week when the viable cell count reaches 2×10^6 to 3×10^6 cells/ml with at least 80% viability.

Sequential Adaptation to SFM:

1. Subculture cells grown in serum-containing medium into a 1:1 ratio of SFM and the original serum-supplemented media with a minimum seeding density of 5×10^5 cells/ml.
2. Incubate cultures until viable cell count exceeds 1×10^6 cells/ml (about one population doubling). Subculture cells by mixing equal volumes of conditioned medium and fresh SFM (1:1).
3. Continue to subdivide the culture in this manner until the serum concentration falls below 0.1%, cell viability is >80%, and a viable cell concentration of $>1 \times 10^6$ cells/ml is achieved.
4. Subculture cells when the viable cell concentration reaches 2×10^6 to 3×10^6 cells/ml (about 4 to 7 days post-planting).

Protocol Notes

- After several passages, viable cell counts of most insect lines should exceed 2×10^6 to 4×10^6 cells/ml. Viabilities should be >85% after approximately 4 to 7 days of culture. At this stage, the culture is adapted to SFM and you should cryopreserve a master cell seed stock for future use (see Protocol 5).

Protocol 5: Preparing a Master Cell Seed Stock

Once a culture is fully adapted to the culture conditions and growth medium, it is essential that you establish a master cell seed stock for each cell line. Master seed stocks should be prepared using the lowest possible passage available. Inventories of 25 to 50 seed stock ampules (4-ml) are generally sufficient; however, if the master stock is to be used for cGMP and/or large-scale production, you may need 100 to 500 ampules. Always store portions of the master cell seed stock in multiple freezers, preferably at different sites, to avoid the possibility of catastrophic loss. With this protocol, you can cryopreserve up to 50 4-ml vials.

Materials List

- automated freezer
- manual freezer tray
- cryovials
- appropriate growth medium (see step 3)

1. Grow desired quantity of cells in suspension using either spinner or shaker culture. Harvest cells in mid-log phase of growth with a viability >90%.
2. Determine the viable cell count, and calculate the required volume of cryopreservation medium required to yield a final cell density of 1×10^7 to 2×10^7 cells/ml.
3. Prepare the required volume of cryopreservation medium.

   Note: For serum-free cultures, you have two choices: prepare a medium consisting of 7.5% DMSO in 50% fresh SFM and 50% conditioned medium (sterile-filtered), or prepare a medium consisting of 100% fresh SFM containing 10% BSA and 7.5% DMSO.

   For serum-supplemented cultures, prepare a fresh medium supplemented with 7.5% DMSO and 10% FBS.
4. Chill the prepared medium and hold at 4°C until use.
5. Centrifuge cells from suspension or monolayer culture medium at $100 \times g$ for 5 min. Decant the supernatant. Resuspend the cell pellet in the chilled cryopreservation medium.
6. Dispense well-mixed aliquots of cell suspension into cryovials according to volumes recommended by the manufacturer.
7. Refrigerate cryovials at 0°C to 4°C for 30 min.
8. Cryopreserve the vials, following standard procedures using a temperature reduction rate of 1°C per minute.

Recovery:

Frozen cells will remain stable indefinitely in liquid nitrogen. Check viability of recovered cryopreserved cells 24 h after storing vials in liquid nitrogen, as follows.

Caution: For safety, always wear a face shield when removing cryovials from liquid nitrogen storage. Doing so will help prevent injury if a vial explodes because of the rapid shift in temperature.

1. Prewarm and equilibrate complete growth medium.
2. Recover cultures from frozen storage by rapidly thawing vials in a 37°C water bath.
3. Wipe or spray ampule exterior with 70% ethanol.
4. Transfer the entire contents of the vial into a shaker or spinner flask containing the prewarmed medium.
5. Inoculate cultures to achieve a minimal viable cell density of 3×10^5 to 5×10^5 cells/ml.
6. Maintain the culture between 0.3×10^6 and 1×10^6 cells/ml for two subcultures after recovery, then return to the normal maintenance schedule.

3. Protocols for Generating a Recombinant Baculovirus

Several molecular biology techniques are available for generating recombinant baculovirus. For optimal results, follow the manufacturer's recommendations for both homologous recombination and site-specific transposition techniques.

Purifying Viral DNA

Several factors are critical for homologous recombination. For homologous recombination, pure viral DNA is required. Techniques to purify viral DNA include phenol extraction (20), cesium chloride purification (20), or affiinity purification with a matrix such as CONCERT™ High Purity Plasmid Purification described in Protocol 6. The choice of protocol depends on the amount of wild-type baculovirus DNA needed.

Protocol 6: Isolation of Bacmid DNA for BAC-TO-BAC® Baculovirus Expression System with the CONCERT High Purity Plasmid Purification System

We have isolated bacmid DNA from DH10BAC with the CONCERT™ High Purity Plasmid Miniprep system using the following protocol. The ~150 kb bacmid (GUS control) was isolated from 1.5 mL overnight culture. This DNA was successfully used in transfection of Sf9 cells. Cells were harvested at 48h and 72h post-transfection and stained according to the BAC-TO-BAC manual. Efficiencies were similar to those observed with transfections using bacmid DNA isolated by other methods.

Inoculation of white colony into miniprep LB kan, gent, tet broth culture:

Inoculate a single, white bacterial colony into 2 ml of LB kan, gent, tet broth (Falcon® 2059 tube.) Place the broth culture in the shaking water bath at 37°C and 250 rpm for a minimum of 16 hours (overnight is fine.)

Isolation of recombinant bacmid DNA:

1. Before beginning: Verify that no precipitate has formed in Cell Lysis Solution (E2.) If the solution E2 is too cold, the SDS will precipitate out of solution. Note: Make sure you have added RNase A to Cell Suspension Buffer (E1.)

2. Column Equilibration: Apply 2 ml of Equilibration Buffer (E4) [600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton X-100] to the column. Allow the solution in the column to drain by gravity flow.

3. Cell Harvesting: Pellet 1.5 ml of an overnight culture. Thoroughly remove all medium.

4. Cell Suspension: Add 0.4 ml of Cell Suspension Buffer (E1) [50mM Tris-HCl (pH 8.0), 10 mM EDTA, containing RNase A at 0.2 mg/ml] to the pellet and suspend cells until homogeneous.

5. Cell Lysis: Add 0.4 ml of Cell Lysis Solution (E2) [200 mM NaOH, 1% SDS]. Mix gently by inverting the capped tube five times. Do not vortex. Incubate at room temperature for 5 min.

6. Neutralization: Add 0.4 ml of Neutralization Buffer (E3) [3.1 M potassium acetate (pH 5.5)] and mix immediately by inverting the tube five times. Do not vortex. Centrifuge the mixture at top speed in a microcentrifuge at room temperature for 10 min. Do not centrifuge at 4°C.

7. Column Loading: Pipet the supernatant from step 12 onto the equilibrated column. Allow the solution in the column to drain by gravity flow. Discard flow-through.

8. Column Wash: Wash the column two times with 2.5 ml of Wash Buffer (E5) [800 mM NaCl, 100 mM Sodium acetate (pH 5.0)]. Allow the solution in the column to drain by gravity flow after each wash. Discard flow-through.

9. Plasmid DNA Elution: Elute the DNA by adding 0.9 ml of Elution Buffer (E6) [1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)]. Allow the solution in the column to drain by gravity flow. Do not force out remaining solution.

10. Plasmid DNA Precipitation: Add 0.63 ml of isopropanol to the eluate. Mix and place on ice for 10 min. Centrifuge the mixture at top speed in a microcentrifuge at room temperature for 20 min. Carefully discard supernatant. Wash the plasmid DNA pellet with 1 ml of ice cold 70% ethanol and centrifuge for 5 min. Carefully and fully pipet off the ethanol wash. Air dry the pellet for 10 min.

11. Purified DNA: Dissolve the pelleted DNA in 40 µl of TE Buffer (TE) [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]. Allow DNA to dissolve for at least 10 min on ice. To avoid DNA shearing, pipet DNA only 1-2 times during resuspension.

Bacmid DNA can be stored at -20°C, but avoid repeated freeze/thawing.

Use 5 µl of this bacmid preparation for transfection of insect cells.

Preparation of Media:

Luria Agar Plates: Miller's Formulation (Premixed formulation of Miller's LB Plates is available: Cat. No. 12945-036)

Note: Use of Lennox L (LB) agar instead of Miller's formulation Luria agar plates will reduce color intensity and may reduce the number of colonies. The use of X-gal instead of Bluo-gal will decrease color intensity.

Component	Amount
SELECT Peptone 140	10 g
SELECT Yeast Extract	5 g
sodium chloride	10 g
SELECT Agar	12 g
distilled water	to a volume of 1 L

Autoclave. Cool solution to 55°C. Antibiotics and supplements are added to the cooled solution.

Component	Stock Soln.	Final Conc.
kanamycin	10 mg/ml (in distilled water)	50 µg/ml
gentamicin	10 mg/ml (in distilled water)	7 µg/ml
tetracycline	5 mg/ml (in ethanol/pH-titrated)	10 µg/ml
IPTG	200 mg/ml (in distilled water)	40 µg/ml
Bluo-Gal	20 mg/ml (in DMSO)	300 µg/ml

Filter-sterilize antibiotics and IPTG. Store at -20°C as aliquots. Mix the agar solution prior to pouring 25 ml per 100 mm petri dish under aseptic conditions. Store agar plates inverted in plastic at 4°C for up to four weeks in the dark.

Protocol 7: Cationic Liposome-Mediated Transfection Using CellFECTIN™ Reagent

DNA can be transfected into insect cells using calcium phosphate coprecipitation, DEAE-dextran-mediated transfection, liposome-mediated transfection, electroporation, and other techniques. Be sure to optimize conditions for your cells. The highest efficiency has been achieved with CellFECTIN Reagent.

For transfection to be efficient, you must use highly purified wild-type baculovirus DNA. To purify wild-type viral DNA, you may use a published procedure or Protocol 6.

This protocol has been optimized for Sf9 cells grown in SFM. CellFECTIN Reagent can be used for cells grown in serum-containing medium as long as you form the lipid/DNA complexes in the absence of serum.

Materials List

- sterile tubes, 12 × 75-mm
- tissue culture plate(s), 6-well
- CellFECTIN Reagent
- 0.5X penicillin/streptomycin/neomycin
- Sf9 or BTI-TN-5B1-4 cells, growing exponentially at a minimum concentration of 5×10^5 viable cells/ml
- Sf-900 II SFM or Express-Five SFM

1. In a 6-well tissue culture plate, seed 9×10^5 Sf9 cells per well in 2 ml of Sf-900 II SFM or 9×10^5 BTI-TN-5B1-4 cells per well in 2 ml of Express-Five SFM (with antibiotics).
2. Incubate the plate at 28°C for at least 1 h to allow cells to attach.
3. In two 12 × 75-mm sterile tubes, prepare the following solutions.

   Solution A: For each transfection, dilute 1 to 2 µg baculovirus DNA and 5 µg transfer vector of choice into 100 µl Sf-900 II SFM or Express-Five SFM without antibiotics.

   Solution B: For each transfection, dilute 1.5 to 9 µl CellFECTIN Reagent into 100 µl Sf-900 II SFM or Express-Five SFM without antibiotics.
4. Add Solution B to the tube containing Solution A, mix gently, and incubate at room temperature for 15 min.
5. While lipid/DNA complexes are forming, wash the Sf9 cells from step 2 once with 2 ml per well of Sf-900 II SFM without antibiotics.
6. Add 0.8 ml Sf-900 II SFM to each tube containing lipid/DNA complexes. Mix gently. Aspirate the wash medium, and overlay the diluted lipid/DNA complexes onto the washed cells.
7. Incubate for 5 h in a 27°C incubator.
8. Remove the transfection mixture. Add 2 ml Sf-900 II SFM or Express-Five SFM (containing antibiotics) per well or dish and incubate at 27°C for 72 h.
9. Harvest the virus from the cell culture medium at 72 h post-transfection.

Protocol 8: Virus Plaque Assay

The infectious potency of a stock of baculovirus is determined by examining and counting plaque formations in an immobilized monolayer culture. Plaquing techniques are generally regarded as the most difficult step in BEVS. Table 5 is provided as a troubleshooting guide for this protocol. Many variations of the basic technique are used, and each provides some advantages depending upon the cell line employed, nature of the recombinant construct, and identification/selection method required. This protocol can be adapted to accommodate variations.

Materials List

- cell culture plates, 6-well
- centrifuge tubes, 12-ml polystyrene (disposable)
- glass bottle, 100-ml sterile (empty)
- Pasteur pipet, sterile, plugged
- sterile pipets, one 1-ml and one 10-ml
- 70°C water bath
- 4% agarose gel or 4% agarose gel with Bluo-gal
- baculovirus supernatant, clarified, cell-free, sterile
- distilled water (sterile), cell-culture-grade
- exponential culture of Sf9, Sf21, or BTI-5B1-4 cells at 5×10^5 cells/ml
- insect cell culture medium: Sf-900 (1.3X) or Grace's Insect Plaquing Medium (2X) plus heat-inactivated FBS. **Note**: For plaquing, Sf900 (1.3X) can be used if cells are grown in any SFM.

1. Under sterile conditions, dispense 2 ml of cell suspension (5×10^5 cells/ml) into each well.
2. Allow cells to settle to bottom of plate. Incubate, covered, at room temperature for 1 h. *If using serum-supplemented media,* transport the plates gently because cells do not adhere tightly to the plate surface.
3. Place the bottle of agarose gel in the 70°C water bath. Place the empty 100-ml bottle and the bottle of 1.3X Sf-900 Insect Medium (or 2X Grace's Insect Medium) in the 37°C water bath.

4. After the 1-h incubation, observe monolayers under the inverted microscope to confirm cell attachment and 50% confluence.
5. Prepare a 10^{-1} to 10^{-8} serial dilution of the harvested viral supernatant by sequentially diluting 0.5 ml of the previous dilution in 4.5 ml of Sf-900 II SFM (or Grace's Insect Cell Culture Medium, Supplemented, without FBS) in the 12-ml disposable tubes. You should have eight tubes containing 5 ml each of the serial dilution from each original virus stock.
6. Move the 6-well plates and the tubes of diluted virus to the hood. Assay each dilution in duplicate.
7. Sequentially remove the supernatant from each well, discard, and immediately replace with 1 ml of the respective virus dilution. Incubate for 1 h at room temperature.
8. Prepare one of the following plaquing overlays:
   - ***Sf-900 plaquing overlay:*** Move bottles from water baths (from step 3) to a sterile hood when the agarose has liquified (after 20 to 30 min). Quickly dispense 30 ml of the 1.3X Sf-900 Insect Plaquing Medium and 10 ml of the 4% Agarose Gel to the empty bottle. Mix gently. Return the bottle of plaquing overlay to the 37°C water bath until use.
   - ***Grace's plaquing overlay:*** Move bottles from water baths (from step 3) to a sterile hood when the agarose has liquified (after 20 to 30 min). Aseptically add 20 ml of heat-inactivated FBS to the Grace's Insect Plaquing Medium and mix. Combine 25 ml of the Grace's Insect Medium supplemented with FBS, 12.5 ml of cell-culture-grade sterile water, and 12.5 ml of the melted 4% Agarose Gel into the sterile empty bottle. Mix gently. Return the plaquing overlay to the 37°C water bath until use.
9. After the 1-h incubation with virus, return the bottle of diluted agarose and the 6-well plates to the hood.
10. Sequentially (from high to low dilution) remove the virus inoculum from the wells and replace with 2 ml of the diluted agarose. Work quickly to avoid desiccation of the monolayer. A Pasteur pipet connected to a vacuum pump easily removes inoculum traces.
11. Let the gel harden 10 to 20 min before moving the plates.
12. Incubate the plates at 27°C in a humidified incubator for 4 to 10 days.
13. Monitor plates daily until the number of plaques counted does not change for 2 consecutive days.

Protocol Notes

- ***Titer:*** To determine the titer of the inoculum employed, count the baculovirus plaques. An optimal range to count is between 3 and 20 plaques per well of a 6-well plate. You can calculate the titer in plaque-forming units/ml using the following formula:

  pfu/ml of original stock = 1/dilution factor × number of plaques × 1/(ml of inoculum/plate)

Identifying the plaques

Because plaques are identified by their phenotype, different screening methods are appropriate for different phenotypes. Most baculoviral plaques fit one of the following four categories:

1. ***Wild-type:*** Plaques from wild-type AcMNPV infections in agarose overlays tend to be highly refractile and near-white in appearance. The plaques can be identified using an inverted light microscope. They will appear as regions of decreased cell density containing many cells with enlarged nuclei. The nuclei will contain many large, dark, angular occlusion bodies.
2. ***Recombinant:*** Plaques from recombinant virus infections (i.e., of co-transfected constructs) can be difficult to locate visually. The milky-grey plaques are small, of low contrast, and often overlooked. This is especially true when they represent a small percentage of the total plaques present. Careful oblique illumination by a high-intensity light source can reveal candidates for quantitation. Marking or scoring the candidates with a felt-tipped pen aids in future recovery. The following methods are useful for identifying plaques from recombinant virus infections:
   - Staining with neutral red solution (Protocol 13) or MTT (0.5 ml of a 1 mg/ml solution per well). Score the wild-type plaques then stain to identify unscored recombinant plaques after staining.
   - Southern blot hybridization of budded virus from the vicinity of a plaque can confirm the presence of the desired gene. Other means (e.g., Western blot or functional assay) are necessary to establish the clone as a successful producer of protein.
3. ***Recombinants expressing chromogenic markers:*** If the recombinant virus bears a reporter gene that produces visible colorimetric reactions, plaques can be detected, counted, and recovered with ease. You can use a vector that contains luciferase or β-galactosidase to help reveal the minority (0.1% to 3%) of successful recombinants. Chromogenic markers also make it easier to quantify plaques in titration studies. Bluo-gal and X-gal reveal recombinant plaques expressing the lacZ gene product by producing a deep blue precipitate.
4. ***Recombinants producing products that can be monitored immunologically:*** These products are distinguished by Western blotting.

TABLE 5. Troubleshooting virus plaque assays.

Problem	Possible Cause	Solution
No or small plaques (other parameters appear fine)	Physical condition of cells is poor	Use cells in mid-log phase growth with viabilities >90%.
	Cell seeding density too high	Decrease seeding density to 10^6 cells per well in a 6-well plate (40% to 50% confluency).
	Inhibition of viral replication cycle due to inadequate nutrition, temperature, or atmospheric conditions	Be sure to make agarose overlay with 1.3X Sf-900 or 2X Grace's Media.
	Misdilution or inactive inoculum	Maintain plates at 27°C in a non-CO_2 atmosphere.
	Note: If the recombinant virus contains a cytotoxic exogenous gene product or inhibits budded virus production, the result is no plaques.	Check that the dilutions were done properly.
Small plaques	Too many plaques on the plate	Inoculate at a higher dilution.
	Premature death of the monolayer due to desiccation of the overlay	Increase humidity in the incubator (*e.g.,* put plates into a container with a damp cloth). Move plates away from wall of incubator. Increase volume of overlay.
	Plasticware may affect insect cell attachment and growth	Evaluate a different style or vendor of plasticware.
Large plaques (hard to identify)	Cell seeding density too low	Increase seeding density to 10^6 cells per well in a 6-well plate (40% to 50% confluency).
	Inhibition of cell growth due to inadequate nutrition, temperature, or atmospheric conditions	Be sure medium is added to the agarose overlay. Maintain plates at 27°C in a non-CO_2 atmosphere.
	Inadequate immobilization of the monolayer	Be sure to completely remove the inoculum.
	Poor gelling of the overlay	Use 4% agarose stock and dilute with medium to 2%.
	Dripping of condensed moisture down the walls of dishes	Allow plates to cool with lids open after adding agarose overlay.
	Gel is detached from the surface of the monolayer	Do not shake plates after overlay is gelled.
Crescent-shaped patches	Monolayer dried partially before addition of either the viral inoculum or gel overlay	Keep cells moist throughout the entire procedure.
	Uneven formation of the monolayer	Allow cells to attach on an even surface.
No plaques or smaller plaques in the center of the plate with larger "smeared" plaques in peripheral regions of the plate	Cell inoculum was distributed by "swirling"	Distribute inoculum by rocking the plate.
Blue regions of β-galactosidase expression too large	Too much chromogenic substrate in overlay	Use a final concentration of 300 µg/ml Bluo-gal.
	Plaques overdeveloped	Develop plates for 3 days and score plaques daily until plaques are distinct.
	Diffusion of dye within gel	Use Bluo-gal to minimize diffusion.
Nearly invisible recombinant plaques while wild-type plaques are quite distinct	Observation for some homologous recombination methods	Develop plates (3 to 7 days) at room temperature to increase the contrast in recombinant plaques. Use a colorimetric marker in the transfer plasmid. Stain the monolayer with neutral red or MTT.
Bubbles on surface of agarose overlay	Bubbles introduced into the molten agarose	Draw up 1 ml more agarose than the procedure requires and do not expel entire contents for the overlay. Touch bubbles with heated sterile pipet or briefly flame surface to pop bubbles.

4. Protocols for Purifying and Producing Recombinant AcNPV and Protein

Protocol 9: Plaque Purification of Recombinant Viral Clones

For homologous recombination, three rounds of plaque purification will ensure generation of a pure recombinant virus stock. Plaque purification is not necessary with the site-specific transposition method.

Materials List

- sealable plastic container (~4 × 8 × 8 in.)
- tissue culture plates, 6-well
- plates with well-developed Occ(-) plaques
- Sf9 or BTI-5B1-4 cells, growing exponentially (viability >95%), at a minimum concentration of 5×10^5 viable cells/ml
- complete serum-free or serum-supplemented insect medium of choice

1. Seed each well with 2 ml Sf9 or BTI-5B1-4 cell suspension, at 5×10^5 viable cells/ml in fresh medium.
2. Mark the plates containing plaques below putative recombinants. For assistance in identifying recombinants, see Identifying the Plaques.
3. Under sterile conditions, remove plugs of the overlay from the selected plaques. Transfer one plug to each well of a multi-well plate.
4. Incubate the plate in a humidified chamber at 27°C.
5. Examine the wells daily for signs of infection and absence of polyhedra.
6. At day 4 or 5, harvest the supernatant. At this point, you may screen and confirm that the recombinant viruses are producing the gene of interest.
7. Following Protocol 8, replaque 10^{-1} to 10^{-3} dilutions of these supernatants. **Note**: It is not necessary to prepare the full range (10^{-1} to 10^{-8}) of serial dilutions. Repeat the plaque purification of the recombinant virus twice and determine virus titers.
8. Amplify confirmed purified producers in either monolayer or shaker infections at a multiplicity of infection (MOI) of 0.1 to 0.01 as described in Protocol 10. Store stocks at 4°C for up to 1 year, protected from light (see Protocol 15).

Protocol 10: Amplifying the Virus Stock

Before you amplify or expand the virus stock, it is essential that you know the titer of your transfection supernatants or plaque-purified virus stocks. Using an MOI of <0.50 will prevent buildup of defective, interfering virus particles. Defective, interfering virus particle buildup is a concern particularly after multiple virus passages and for virus produced under serum-free conditions. With a low MOI, cell cultures will continue to grow post-infection. To prevent rate-limiting nutritional problems that may result in decreased viral production and titers during expansion of virus stocks, follow the guidelines for maximum viable cell densities in table 6.

1. Infect a suspension or monolayer culture in mid-exponential growth at an MOI of 0.01 to 0.1 according to the following formula:

$$\text{Inoculum required (ml)} = \frac{\text{Desired MOI (pfu/cell)} \times \text{(total number of cells)}}{\text{Titer of viral inoculum (pfu/ml)}}$$

   Note: At 48 h post-infection usually yields a 2-log amplification.

   Example: Infect a 50-ml culture of Sf9 cells at 2×10^6 cells/ml with 0.5 ml of a viral stock containing 2×10^7 pfu/ml to obtain an MOI of 0.10.
2. Harvest the culture 24 to 48 h post-infection. Titer the virus stock by plaque assay (see Protocol 8).
3. Repeat steps 1 and 2 until virus stock has a confirmed titer of 1×10^7 to 1×10^8 pfu/ml.
4. Store the virus stocks at 4°C for up to 1 year, protected from light (see Protocol 15).

Protocol 11: Identifying Plaques by Neutral Red Staining

Incubation times and the amount of stain used vary depending on the plaquing medium and dishes. ***Handle the plates gently*** throughout any staining procedure as the monolayer is easily disrupted.

TABLE 6. Recommended maximum infection densities for the production of rAcNPV or recombinant products

	Serum-free media				Serum-supplemented media			
	Monolayer culture		Suspension culture		Monolayer culture		Suspension culture	
Cell line	rAcNPV	Recomb. product	rAcNPV	Recomb. product	rAcNPV	Recomb. product	rAcNPV	Recomb. product
Sf-9	0.50–1.0	0.50–1.0*	1.5–2.0	2.0–3.0	0.5	0.5	1.0–1.5	1.0–2.0
Sf-21	0.50–1.0	0.50–1.0	1.0–1.5	1.0–2.0	0.5	0.5	1.0	1.0–1.5
Tn-368	0.25–0.50	0.25–0.50	0.5–1.5	1.0–2.0	0.25–0.5	0.25–0.50	0.5–1.0	1.0
BTI-TN-5B4-1	0.25–0.50	0.25–0.50	0.5–1.5	1.5–2.0	NA	NA	NA	NA

* Numbers are viable cells/ml × 10^6.

Materials List

- distilled water, cell-culture-tested
- neutral red staining solution (3.3 g/L)
- plates with developed Occ⁻ plaques

1. For plaque purification, score all visible plaques with a felt-tip pen. This will make it easier to identify potential producers of recombinant product.
2. Freshly prepare a 0.1% (w/v) neutral red stain solution in cell-culture-grade water.
3. To each well containing 2 ml of plaquing overlay, add 0.5 ml of 0.1% neutral red solution. Incubate for 1 to 2 h at room temperature.
4. Gently remove excess stain with a pipet or blotter.
5. Plates yield clear plaques in a nearly clear gel against a russet background. Unscored plaques made visible by staining are potential recombinants.

Protocol 12: Optimizing Virus Stock Production

This protocol can be used to optimize and produce high-quality, high-titer master or working virus stocks.

Materials List

- complete serum-free or serum-supplemented medium of choice
- high-titer rAcNPV stock (>1 × 10^7 pfu/ml); and
- shake or spinner flasks

1. Set up and inoculate 15 replicate serum-free or serum-supplemented suspension cultures in triplicate as described in Protocol 3.
2. Grow cultures for 2 to 3 days until they are in mid-exponential growth (16- to 24-h doubling times) and have attained the cell densities recommended for infection in table 6.

   Note: If the cell culture exceeds the density recommended in table 5, dilute the cell culture before infection with up to 50% fresh media. Be sure, however, that the total volume does not exceed that recommended in table 4.
3. Infect triplicate flasks at each of the following MOIs: 0.01, 0.05, 0.10, and 0.50 (see Protocol 10, step 1, to determine virus inocula required at each MOI). Maintain one set of flasks as uninfected growth controls.
4. Sample flasks 24, 48, and 72 h post-infection. Compare morphologies and cell densities of infected cultures against noninfected controls to confirm progress of infection. Determine total and viable cell counts and store 1 to 5 ml of clarified, sterile virus from each sample at 4°C.
5. Determine the virus titer of each sample by plaque assay (Protocol 8).
6. Select the optimal MOI and the harvest time that produced the highest combination of virus titer and culture viability >80%. Produce a large quantity of working and/or master virus stock using these infection parameters.
7. Store working virus stocks at 4°C and master virus stocks at –70°C or in liquid nitrogen, as recommended in Protocol 15.

Protocol 13: Harvesting the Virus

Extracellular virus, or budded virus, begins accumulating in the growth medium ~8 to 10 h post-infection and continues accumulating through ~20 to 30 h. With a synchronized infection (MOI >4.0), budded virus production is complete at ~30 h post-infection. There is little or no benefit to longer incubations. Budded virus with functional titers is possible at 12 h post-infection. Harvesting before the lytic phase when the cell viabilities are >90% will minimize contamination by cell debris, metabolic waste products, and proteases. In non-synchronous infections (MOI <4.0), budded virus can be harvested through approximately 48 h post-infection.

With this protocol, loss of virus titer will be minimal (<10%). Further purification of the virus is not usually necessary.

Materials List

- centrifuge tubes
- 0.2-µm low-protein binding filter unit

1. Decant or aspirate the growth medium containing virus from the culture into centrifuge tubes.
2. Centrifuge at 250 × g for 5 min to remove cells and large debris.

   For suspension cultures: If desired, centrifuge a second time at 1,000 × g for 20 to 30 min.
3. Sterile filter, if desired, through a 0.2-µm low-protein binding filter.
4. Store virus as recommended in Protocol 15.

Protocol 14: Concentrating the Virus

To produce viral DNA or to achieve an otherwise unobtainable MOI (>10.0), use this protocol to concentrate the virus from growth medium. The supernatant must be harvested from a nonlytic, serum-free culture.

Materials List

- ultracentrifuge tubes, 38-ml polyallomer
- 0.2-µm low-protein binding filter unit
- virus stock to be concentrated
- sucrose solution: 25% sucrose in 5 mM NaCl, 10 mM EDTA
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (pH 6.2)

1. Load 33 ml of virus stock into each of six 38-ml polyallomer ultracentrifuge tubes.
2. Underlay the virus stock with 3 ml of sucrose solution per tube.
3. Centrifuge at 80,000 × g for 75 min at 4°C.
4. Decant the supernatant, removing as much from the walls of the tube as possible. A relatively pure viral pellet will be translucent white, with faint blue color near the edges. Less pure pellets display increasing opaqueness and size; their color ranges from pale yellow to light brown as contamination increases.
5. Resuspend pellets in 0.5 to 5 ml D-PBS or cell growth medium. Resuspension may require some effort. Allow sufficient time after resuspension for the cells to disrupt completely. Filter through a 0.2-µm filter. Store at 4°C.

Protocol 15: Storing the Virus

Virions are quite stable in standard serum-supplemented growth media. They maintain their integrity and infectious competency for days at elevated temperatures, weeks at room temperature, and months to years at 4°C.

If virions will be stored for longer than 3 months under serum-free conditions, add 0.1% to 1% BSA to stabilize the virus. Store the virus stocks in polypropylene containers or siliconized glassware to prevent nonspecific binding of virus. They should be retitered periodically if used as inoculates. Loss in virus titer will be minimal (<10%) with this protocol.

Materials List

- centrifuge tubes
- sterile cryotubes (or other large-volume container suitable for freezing)
- 0.2-µm low-protein binding filter unit (optional)
- virus supernatant

1. Aseptically transfer virus-containing supernatant to a sterile, capped centrifuge tube. Centrifuge 5 min at 500 × g. Decant or transfer the virus-containing supernatant to a fresh tube(s).
2. Sterile filter, if desired, through a 0.2-µm, low-protein binding filter.
3. Dispense the clarified, sterile-filtered supernatant into cryotubes (or suitable larger volume containers).
4. Store the virus stocks at 4°C, protected from light. For long-term storage at 4°C, –70°C, or in liquid nitrogen, we recommend adding BSA to a final concentration of 0.1% to 1%.

Protocol 16: Optimizing Heterologous Protein Production

The first step toward successful infection of insect cells with either wild-type or recombinant baculovirus is ensuring that the culture will not be rate limited by nutritional factors (*i.e.,* amino acid or carbohydrate utilization) or environmental factors (*i.e.,* pH, dissolved O_2, temperature). Cultures should be infected while in the mid-logarithmic phase of growth with an established MOI. The optimal MOI varies by cell line and the relative infection kinetics of the virus isolate or clone employed. A dose response (or MOI) should be established for each virus, medium, reactor, and cell line employed. This information will enable you to determine optimal infection parameters for production of virus or recombinant product.

When producing non-occluded virus stock (recombinant or wild-type), infect the suspension culture at a cell density of 1×10^6 to 2×10^6 cells/ml with an MOI of 0.01 to 0.1 (see Protocol 12 and table 5). To express recombinant gene products, MOIs of 0.5 to 10 are commonly employed. Standard serum-supplemented media used for virus infection are rate limiting if the cells are infected at densities $>2 \times 10^6$ cells/ml. However, with Sf-900 II SFM, suspension cultures have been successfully infected at 2×10^6 to 3×10^6 cells/ml, and successes have been reported at $>4 \times 10^6$ cells/ml (21).

The BEVS recombinant gene product may or may not be secreted. Maximum expression is usually observed between 30 and 72 h for secreted proteins and between 48 and 96 h post-infection for nonsecreted proteins. It is important to determine the expression kinetics for each product, as many proteins (secreted or nonsecreted) may be degraded by cellular proteases released in cell culture.

To express some recombinant products and/or rAcNPV, you may need to protect the recombinant product or virus from proteolysis by supplementing serum-free cultures post-infection with 0.1% to 0.5% FBS or BSA. Protein-based protease inhibitors are generally less expensive and more effective than many synthetic protease inhibitors.

This protocol is suitable for determining both the optimal MOI and harvest time for the production of your recombinant product.

Materials List

- complete serum-free or serum-supplemented medium of choice
- high-titer rAcNPV stock ($>1 \times 10^7$ pfu/ml)
- shake or spinner flasks

1. Set up and inoculate 15 replicate serum-free or serum-supplemented suspension cultures as described in Protocol 3.
2. Grow cultures for 2 to 3 days until they are in mid-exponential growth (16- to 24-h doubling times) and have attained the cell densities recommended for infection in table 6.

   Note: If the cell culture exceeds the density recommended in table 5, dilute the cell culture before infection with up to 50% fresh media. Be sure, however, that the total volume does not exceed that recommended in table 4.
3. Infect triplicate flasks at each of the following MOIs: 0.50, 1.0, 5.0, and 10.0 (see Protocol 10, step 1, to determine virus inocula required at each MOI). Maintain one set of flasks as uninfected growth controls.
4. Sample flasks 24, 48, 72, and 96 h post-infection. Compare morphologies and cell densities of infected cultures against non-infected controls to confirm progress of infection. Determine total and viable cell counts.

   Note: Optimal product expression is often between 48 and 72 h post-infection, so you may want to sample cultures every 8 to 12 h after 24 h post-infection.
5. Store cell pellet from 1 to 5 ml of cell suspension at -20°C (for nonsecreted products) or 1 to 5 ml of clarified supernatants at 4°C (for secreted products).
6. Assay cell pellets or supernatant samples for recombinant product yields and/or activity.
7. Select the optimal MOI and the harvest time that produced the highest combination of product yield/activity and quality/homogeneity.
8. Scale up the production of recombinant product using these infection parameters. Reconfirm optimal harvest time after scale-up.

5. Purifying Recombinant Proteins

The following criteria are important to consider when selecting a purification protocol:

- ***Scale of Expression:*** Protocols efficient in small scale may not be efficient in large scale.
- ***Nature of the Product Expressed:*** Consider using immunoaffinity chromatography when a low-cost source of pure antibody exists for the protein.
- ***Growth Medium:*** Serum-free culture supernatants harvested from infected cultures before significant cell lysis occurs may have recombinant product as a majority (upwards of 95%) of the total protein complement.
- ***Product Application:*** Practical and/or regulatory demands may determine the purification approach.

When designing a purification protocol, consider the impact of each of the following:

Use of Hydrolysates, Extracts, Lipids, and Sterols: Many of these media supplements are not defined. They can have some unpredictable interactions with both the protein of interest and/or the chromatographic technique. Affinity chromatography generally will eliminate problems related to nonspecific interactions. If you cannot use affinity chromatography, try to eliminate these media components in the first purification step (*i.e.,* diafiltration with a buffer exchange step).

Use of Pluronic F-68 Co-polymer: Most serum-free insect cell culture media contain surface active agents such as Pluronic F-68 that can cause problems during certain purification procedures. Pluronic F-68 may exist in culture as a wide range of polymeric structures dependent upon concentration; pH; temperature; and the presence of other surfactant(s), detergents, lipids, sterols, or polar molecules. Although Pluronic F-68 does not interfere with many chromatographic and precipitation techniques, it will precipitate in the presence of high salt concentrations. Before further processing that may involve high salt concentrates, such as $(NH_4)_2\ SO_4$ precipitation or hydrophobic interaction chromatography (HIC), diafiltrate with a buffer exchange step.

Presence of a Cystine Protease: Ambient medium of baculovirus infected cells may contain a cystine protease (22,23). Proteolysis is a serious issue in serum-free cultures. Because SFM are low in protein or protein-free, they provide little competitive substrate for the protease activity. Secreted proteins have demonstrated a variable sensitivity to ambient proteases. Researchers have examined a variety of protease inhibitors with variable success. A report using pCMBS (p-chloromercuribenzene) appears promising (24). The best way to reduce the chance of significant proteolysis is to keep post-infection culture supernatants refrigerated, to harvest the product before significant cell lysis occurs, and to process the product as soon as possible after harvest. Addition of 0.1 to 1% BSA can provide a competitive substrate for the protease.

Secreted Proteins: Proteins expressed in the baculovirus expression vector system accumulate extracellularly in the growth medium as secreted proteins, or intracellularly. Nascent proteins with absent or aberrant signal sequences may not process normally and, as a result, may be nonsecretory. Protocols for the purification of intracellular product begin with the physical or chemical disruption of cells, followed by isolation procedures.

To clarify secreted proteins, use settling, centrifugation, or filtration. Further processing of the supernatant can include gel filtration, chromatography, and precipitation.

Purification from Sf-900 II SFM or Express-Five SFM

The chief advantage to using SFM for culture of insect cells is that purification protocols are simplified because contaminating proteins are reduced. One disadvantage is the possible proteolytic degradation of proteins when concentrating product.

Purifying Secreted Proteins

Use the following guidelines to purify secreted proteins. To simplify purification protocols and prevent problems in later steps, we recommend a thorough buffer exchange or washing early in the purification such as at the concentration step.

Removing Cells

Supernatants should be clarified before further processing.

For small-scale cultures:

Centrifugation for 5 min at 1,000 × g may be sufficient. You can also remove the virus by ultracentrifugation at 80,000 × g for 75 min.

For large liquid volumes:

You have several options for removing cells in large liquid volumes. You can clarify the supernatant with cartridge membranes. The advantage of cartridge membranes is that they can be sterilized in place. You can use ultrafiltration membranes, but these tend to foul. For cross-flow, tangential-flow and hollow-fiber systems, you can use microporous filter membranes. These offer a higher flux rate and are less likely to foul.

Removing Baculovirus

Options for removing baculoviruses from small- or large-scale culture supernatants include membrane filtration apparatus and chromatographic techniques such as anion exchange. For more information on virus removal and inactivation, see Grun et al. (25).

Concentrating the Product

The product can be concentrated by dialysis, membrane filtration, or precipitation followed by centrifugation. For dialysis and membrane filtration, use a membrane with a 10-kDa or greater cut-off to allow media components to pass into the filtrate. The membrane may have to be smaller if the product of interest is below 50 kDa. Bear in mind that molecular weight cut-off is a nominal value. Some products with molecular weights greater than the cut-off value may pass through the membrane. The amount that passes through depends on the membrane pore distribution and the nominal molecular weight cut-off value. During the

concentration procedure, addition of protease inhibitors may diminish proteolytic and glucosidase activity. Cell culture supernatants should be concentrated 10 to 20 times, resuspended in buffer, and reconcentrated to remove media components. After concentration of sample, protein is purified as necessary. When possible, affinity chromatography is used. Many columns and resins are available depending on your needs (26-31).

For concentration by precipitation from serum-free media, use polyethylene glycol (PEG) (32). Ammonium sulfate precipitation is not recommended for recovering proteins from SFM.

Purifying Intracellular Proteins

To harvest intracellular products, cells are lysed most commonly by sonication. Cells are spun down at 200 to 400 $\times$ *g* for 10 min, the supernatant is removed. The pellet is resuspended in a lysing buffer, usually containing sucrose up to 0.3 M, and protease inhibitors such as pepstatin or phenylmethylsulfonyl fluoride (PMSF).

Staudacher (33) employed a simple method of sonication lysis of pelleted cells in 0.025 M sucrose. Cells, on ice, are repeatedly sonicated for short periods (~10 s) after which cellular debris is removed by centrifugation. Another method for lysing cells without mechanical force has been described by Emery (34).

If cells are lysed with detergent, remove detergent after lysis to minimize its interference with further purification steps. After cell lysis, samples are usually concentrated before further purification.

6. References

1. Summers, M. and Anderson, D. (1972) *J. Virol. 9,* 710.
2. Matthews, R.E F. (1982) *Interviology 17*, 1.
3. Longworth, J.L. (1983) *A Critical Appraisal of Virus Taxonomy,* Matthews, R.E.F. (ed.), CRC Press, Boca Raton, FL, p. 123.
4. Smith, G.E., Summers, M.D. and Fraser, M.J.(1983) *J. Molecular and Cellular Biology 3*, 2156.
5. Smith, G.E., Summers, M.D., and Fraser, M.J. (1983) *Mol. Cell. Biol. 3*, 2156.
6. Ignoffo, C.M. (1975) *Baculoviruses for Insect Pest Control: Safety Considerations*, Summers, M., Engler, R., Falcon, L.A. and Vail, P.V. (eds.), American Society for Microbiology, Washington, DC, p. 52.
7. Kitts, P.A., Ayres, M.D. and Possee, R.D. (1990) *Nucleic Acids Res. 18*, 5667.
8. Hartig, P.C. and Cardon, M.C. (1992) *J. Virol. Methods 38*, 61.
9. Kitts, P.A. and Possee, R.D. (1993) *Biotechniques 14*, 810.
10. Luckow, V.A., Lee, S.C., Barry, G.F., and Olins, P.O. (1993) *J. Virol. 67*, 4566.
11. Anderson, D., Harris, R., Polayes, D., Ciccarone, V., Donahue, R., Gerard, G., and Jessee, J. (1996) *Focus 17*, 53.
12. Polayes, D., Harris, R., Anderson, D., and Ciccarone, V., (1996) *Focus 18*, 10.
13. Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., and McCawley, P. (1977) *In Vitro 13*, 213.
14. Hink, W.F., Thomsen, D.R., Davidson, D.J., Meyer, A. L. and Castellino, F.J. (1991) *Biotechol. Prog. 7*, 9.
15. Mitsuhashi, J. (1982) *Advances in Cell Culture 2*, 133.
16. Hink, W.F. and Bezanson, D.R. (1985) *Tech. in Life Sci., Vol. C111*, Elsevier Scientific Publishers Ireland Ltd., County Clare, Ireland, p. 19.
17. Inlow, D., Shauger, A., and Maiorella, B. (1989) *J. Tissue Cult. Methods 12*, 13.
18. Barile, M.F. and Kern, J. (1971) *Proc. Soc. Exp. Biol. Med. 138*, 432.
19. Hay, R.J. (1988) *Analytical Biochem. 171*, 225.
20. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
21. Weiss, S.A., Whitford, W.G. and Godwin, G. (1991) *Proceedings of the Eighth International Conference on Invertebrate and Fish Tissue Culture*, Fraser, M.J. (ed.), Tissue Culture Association, Columbia, Maryland, p. 153.
22. Yamada, K., Nakajima, Y., and Natori, S. (1990) *Biochem. J. 272*, 633.
23. Vernet, T., Tessier, D.C., Richardson, C., Laliberte, F., Khouri, H.E., Bell, A.W., Storer, A.C., and Thomas, D.Y. (1990) *J. Biol. Chem. 265*, 16661.
24. Yamada, K., Nakajima, Y., and Natori, S. (1990) *Biochem. J. 272*, 633.
25. Grun, J. B., White, E.M., and Sito, A.F. (1992) *BioPharm 5*, 22.
26. Staudacher, E., Kubelka, V. and Marz, L. (1992) *European Journal of Biochemistry 207*, 987.
27. Wimalasena, R.L. and Wilson, G.S. (1992) *LC•GC 10*, 223.
28. Majors, R.E. and Hardy, D. (1992) *LC•GC 10*, 356.
29. Wathen, M.W., Brideau, R.J., and Thomsen, D.R. (1989) *J. Infectious Dis. 159*, 255.
30. Gavit, P., Walker, M., Wheeler, T., Bui, P., Lei, S., and Weickmann, J. (1992) *BioPharm 5*, 28.
31. Frenz, J., Hancock, W.S., and Wu, S. (1992) *LC•GC 10*, 668.
32. Ingham, K.C. (1990) *Meth. Enzym. 182*, 301.
33. Emery, V.C. and Bishop, D.H.L. (1987) *Protein Engineering I*, 359.
34. Drake, L. and Barnett, T. (1992) *BioTechniques 12*, 645.

7. Related Products

Product	Size	Cat. No.
BAC-TO-BAC Products:		
Plasmid pFASTBAC1 Expression Vector pFASTBAC DNA pFASTBAC-gus DNA (4 ng) Manual	10 µg	10360-014
pFASTBAC HT Expression Vectors pFASTBAC HTa pFASTBAC HTb pFASTBAC HTc pFASTBAC HT-CAT (15 ng) Ni-NTA resin (10 ml) Disposable column (1) Manual	10 µg each	10584-027
pFASTBAC DUAL Expression Vector pFASTBAC DUAL DNA pFASTBAC DUAL Control DNA (4 ng) Manual	10 µg	10712-024
MAX EFFICIENCY DH10BAC Competent Cells	5 x 0.1 ml	10361-012
CELLFECTIN Reagent	1 ml	10362-010
BAC-TO-BAC Expression Systems:		
BAC-TO-BAC Baculovirus Expression System pFASTBAC1 Expression Vector (1 each) MAX EFFICIENCY DH10BAC Competent Cells (5 x 0.1 ml) CELLFECTIN Reagent (1 ml)	5 reactions	10359-016
BAC-TO-BAC HT Baculovirus Expression System pFASTBAC HT Expression Vectors (1 each) MAX EFFICIENCY DH10BAC Competent Cells (5 x 0.1 ml) CELLFECTIN Reagent (1 ml)	5 reactions	10608-016
Molecular Genetics Media and Antibiotics:		
Ampicillin Sodium Salt, lyophilized	5 ml	13075-015
Gentamicin Reagent Solution (10 mg/ml), liquid	10 ml	15710-015
Kanamycin Sulfate (100X), liquid	100 ml	15160-013
LB Agar, powder	500 g	22700-025
LB Broth (1X), liquid	500 ml	10855-013
Luria Agar, powder	100 g	12945-028
S.O.C. Medium	10 × 10 ml	15544-018
Terrific Broth	100 g	22711-014
SELECT Peptone 140	500 g	30392-021
SELECT Yeast Extract	500 g	30393-029
SELECT Agar	500 g	30391-023
Molecular Biology Products:		
1 Kb DNA Ladder	250 µg	15615-016
λ DNA/*Hin*d III Fragments	500 µg	15612-013
SUBCLONING EFFICIENCY DH5α Competent Cells	2 ml	18265-017
Taq DNA Polymerase, Recombinant	100 units	10342-053
Concert High Purity Miniprep System	25 reactions 100 reactions	11449-014 11449-022
Concert High Purity Midiprep System	25 reactions 50 reactions	11451-010 11451-028
Concert High Purity Maxiprep System	10 reactions 25 reactions	11452-018 11452-026

Product	Size	Cat. No.
Reagents:		
4% Agarose Gel	40 ml	18300-012
Agarose	100 g	15510-019
Bluo-gal	100 mg	15519-010
Buffer-Saturated Phenol	100 ml	15513-039
Ethidium Bromide	1 g	15582-018
Ethylenediaminetetraacetic Acid	100 g	15576-010
IPTG	1 g	15529-019
MUG	100 mg	10215-010
Phosphate-Buffered Saline (PBS), pH 7.4 (1X)	500 ml	10010-015
Phosphate-Buffered Saline (PBS), pH 7.4 (10X)	500 ml	70011-036
10% SDS Solution	4 × 100 ml	15553-019
10X TAE Buffer	1 L	15558-042
1 M Tris-HCl, pH 8.0	1 L	15568-026
X-Glucuronide	100 mg	10214-013
Nucleic Acid Purification Rack	each	11494-010
Insect Media:		
Fetal Bovine Serum, qualified, heat-inactivated	100 ml	16140-014
Grace's Insect Medium (1X), liquid	500 ml	11595-022
IPL-41 Insect Medium (1X), liquid	500 ml	11405-024
Neutral Red Solution	100 ml	15330-012
Penicillin-Streptomycin, liquid	100 ml	15070-014
PLURONIC® F-68, 10% (100X)	100 ml	24040-016
Sf-900 II SFM (1X), liquid with L-glutamine	500 ml	10902-013
Sf-900 II SFM (1X), methionine and cystine-free	500 ml	21012-018
Sf-900 II SFM (1.3X), liquid with L-glutamine	100 ml	10967-016
TC-100 Insect Medium, powder	10 × 1 L	11600-061

A. Applications Data for Insect Cell Lines Grown in Serum-Free Medium

Monolayer cultures of Sf9, Sf21, and Tn-368 cells in Grace's supplemented medium plus 10% heat-inactivated FBS were adapted to suspension culture as described in Protocol 2, and then to serum-free growth in Sf-900 II SFM using the direct adaptation method described in Protocol 4. The monolayer BTI-TN-5B1-4 culture was adapted to growth in Sf-900 II SFM, then to suspension culture in the same medium, and finally to EXPRESS-FIVE SFM.

Following a minimum of 10 consecutive passages in each medium, the four cell lines were seeded in 35- to 150-ml shake flasks or spinner cultures at 2×10^5 to 3×10^5 viable cells/ml. Cultures were incubated at 27°C with stirring speeds of 90 to 100 rpm for spinner flasks and 135 to 150 rpm for shaker flasks. Results in table 7 represent maximum cell densities in small-scale suspension cultures on days 4 to 7 post-planting.

TABLE 7. Maximum cell densities in small-scale suspension culture.

Cell line	Growth Medium: Grace's TNM-FH + 10% FBS (viable cells/ml $\times 10^6$)	Sf-900 II SFM (viable cells/ml $\times 10^6$)	EXPRESS-FIVE SFM (viable cells/ml $\times 10^6$)
Sf9	4 to 6	8 to 12	—
Sf21	3 to 5	5 to 7	—
Tn-368	2 to 3	3 to 5	—
BTI-TN-5B1-4	—	3 to 4	4 to 5

Comments

- Tn-368 cells usually maintain their characteristic spindle morphology under suspension conditions if the growth medium is maintained within optimal pH and osmolality ranges.
- Unlike the Sf9 or Sf21 cell lines, Tn-368 and BTI-TN-5B1-4 cultures often die rapidly upon reaching maximum cell density and are difficult to recover if viabilities drop below 50%. To avoid problems, cultures of Tn-368 and BTI-TN-5B1-4 cells should be split frequently while in mid-exponential growth.

Expression of Recombinant Protein in Small-Scale Culture

Shake flask cultures (50- to 100-ml) of Sf9, Tn-368, and BTI-TN-5B1-4 cells were adapted to growth in serum-free or serum-supplemented medium. The cultures were infected with rAcNPV (Clone VL-941) expressing recombinant b-galactosidase at the following densities and MOIs:

Sf9 cells:	2.5×10^6 viable cells/ml	MOI = 5.0
Tn-368 cells:	1.0×10^6 viable cells/ml	MOI = 5.0
BTI-TN-5B1-4 cells:	1.5×10^6 viable cells/ml	MOI = 4.0

Cultures were incubated post-infection at 27°C with a stirring speed of 135 rpm. Recombinant β-galactosidase activity was monitored through day 4 or 5 post-infection for each culture. Results are shown in table 8.

Comments

- Recommended infection densities are lower for the Tn-368 and BTI-TN-5B1-4 cell lines because in serum-free suspension culture these cells generally attain lower maximum densities (5×10^6 to 6×10^6 viable cells/ml) than Sf9 cells (8×10^6 to 12×10^6 viable cells/ml). Infect cultures while in mid-exponential growth (population doubling times of 16 to 24 h) at cell densities no greater than 40% of the maximum normally observed for optimal expression.
- Recombinant protein expression varies for different proteins, and the optimal cells for each protein can vary.

Growth and Expression of Recombinant Proteins in Large-Scale Culture

For scale up of a recombinant product using BEVS technology, it is important to determine whether the medium (serum-supplemented or serum-free) will adequately support scale-up, as well as downstream processing considerations (*i.e.,* cell separation and product purification).

The data in table 9 compare results of pilot-scale cell growth and expression of recombinant proteins in Sf-900 II SFM versus serum-supplemented medium. Recombinant product yields reached or exceeded levels obtained under small-scale conditions. Product yields were up to 10-fold higher with Sf-900 II SFM than those produced under serum-supplemented conditions and display acceptable glycosylation or bioactivity.

Comparison of rAcNPV Titer in Small-Scale Suspension Culture

Shake flask cultures (75-ml) of Sf9 and BTI-TN-5B1-4 cells were adapted to growth in various media. The cultures were infected with rAcNPV (Clone VL-941) expres-sing recombinant β-galactosidase. Triplicate cultures for each were infected at 1×10^6 viable cells/ml at an MOI of 0.10.

Cultures were infected at 27°C with a stirring speed of 135 rpm. The cultures were sampled at 24, 48, and 72 h post-infection. Clarified supernatant samples were titered by plaque assay. Results are shown in table 10.

Comments

- For BTI-TN-5B1-4 cultures, maximum rAcNPV titers were almost 2 logs lower than Sf9 cells. It is not unusual for BTI-TN-5B1-4 cells to produce virus stocks 1 to 3 logs lower than comparable Sf9 or Sf21 cultures. To counteract this, maintain and produce your working rAcNPV stocks in Sf9 or Sf21 cells and use the BTI-TN-5B1-4 cell line for expression of recombinant products.

TABLE 8. β-galactosidase expression in small-scale suspension culture.

	Sf9 cells		Tn-368		BTI-TN-5B1-4 cells	
Days post-infection	Grace's TNM-FH + 10% FBS	Sf-900 II SFM	Grace's TNM-FH + 10% FBS	Sf-900 II SFM	Sf-900 II SFM	EXPRESS-FIVE SFM
1	—	—	6	8	9	9
2	—	—	—	—	16	28
3	95	254	25	61	79	252
4	276	550	—	—	499	798
5	198	583	26	69	—	—

Note: Data are units β-gal/ml × 10^3.

TABLE 9. Pilot-scale recombinant protein expression in cells cultured.

		Expression level	
Recombinant protein	Bioreactor	In Sf-900 II SFM	In serum control
α-Galactosidase	2-L Celligen 30-L Chemap airlift	4,700 U/ml 5,040 U/ml	2,500–5,000 U/ml
β-Galactosidase	5-L Celligen	240,000 U/ml	150,000 U/ml
Erythropoietin	2-L Celligen 5-L Celligen	7,800 U/ml 6,500 U/ml	1,000–2,000 U/ml
Hantaan S nucleocapsid	5-L Celligen	5-fold higher than serum control*	
Human choriogonadotropin	5-L Celligen	8,192–8,345 ng/ml	768–1,075 ng/ml in monolayer
Leukemia inhibitory factor	10-L Braun	9 µg/ml	Same as *E. coli*
rVP6, rotavirus capsid protein	5-L Celligen	118 µg/ml	20 µg/ml in IPL-41 with 10% FBS

*Specific product yield not provided.

TABLE 10. rAcNPV titers in small-scale suspension culture.

		Virus titer post-infection (pfu/ml)		
Cell line	Medium	24 h	48 h	72 h
Sf9	Grace's TNM-FH supplemented with 10% FBS	1×10^8	5×10^8	6×10^8
Sf9	Sf-900 II SFM	5×10^7	3×10^8	4×10^8
BTI-TN- 5B4-1	EXPRESS-FIVE SFM	2×10^5	6×10^6	5×10^6

Notes:

INSIDE BACK COVER

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 603 7229
Email: tech_service@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
Email: eurotech@invitrogen.com

International Offices:
Argentina 5411 4556 0844
Australia 1 800 331 627
Austria 0800 20 1087
Belgium 0800 14894
Brazil 0800 11 0575
Canada 800 263 6236
China 10 6849 2578
Denmark 80 30 17 40

France 0800 23 20 79
Germany 0800 083 0902
Hong Kong 2407 8450
India 11 577 3282
Italy 02 98 22 201
Japan 03 3663 7974
The Netherlands 0800 099 3310
New Zealand 0800 600 200
Norway 00800 5456 5456

Spain & Portugal 900 181 461
Sweden 020 26 34 52
Switzerland 0800 848 800
Taiwan 2 2651 6156
UK 0800 838 380
For other countries see our website

www.invitrogen.com

308-CC
Date 022702