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David W. Murhammer *Editor*

# Baculovirus and Insect Cell Expression Protocols

*Third Edition*

Humana Press

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# Baculovirus and Insect Cell Expression Protocols

**Third Edition**

Edited by

**David W. Murhammer**

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# Preface

Baculoviruses, which are a group of viruses that infect invertebrates, were first "discovered" in diseased silk worms in the 1500s, although the viral nature of this disease was not demonstrated until 1947. Subsequently, hundreds of other baculoviruses have been discovered. For example, the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was first isolated from the alfalfa looper (i.e., *A. californica*) insect species. AcMNPV is the most widely used and best characterized baculovirus and is known to infect many insect species in addition to *A. californica*, including *Spodoptera frugiperda* (fall armyworm) and *Trichoplusia ni* (cabbage looper). Furthermore, AcMNPV is the baculovirus that is usually used to produce recombinant baculoviruses for subsequent recombinant protein synthesis.

Shangyin Gao and Thomas Grace independently established the first continuous insect cell lines in the late 1950s and early 1960s. In the 1960s and 1970s insect cell culture was primarily used as a model to study insect metabolism and for the in vitro synthesis of baculoviruses for potential use in insect control (i.e., as a biopesticide). The widespread use of insect cell culture, however, did not occur until the baculovirus expression vector system (BEVS) was independently developed in the Max D. Summers and Lois K. Miller laboratories in the early 1980s. The BEVS takes advantage of the very strong polyhedrin promoter found in the AcMNPV genome whose natural product (polyhedrin protein) is nonessential in insect cell culture. Thus, the BEVS involves using the polyhedrin promoter to drive foreign protein expression and provides the means to express high levels of recombinant proteins in a relatively short time. Note that there have been many extensions of this basic principle, many of which are described in this book. In brief, some advantages of the BEVS are (1) ease of constructing a recombinant baculovirus (compared to isolating stably transformed cells), (2) potentially high expression levels, and (3) the ability of host insect cells to properly process proteins in a manner similar to mammalian cells. Thus, the BEVS provides a recombinant protein expression system intermediate between bacteria (e.g., *E. coli*) and mammalian cells (e.g., CHO cells) in terms of expression levels, cost, and ability to perform complex protein modifications. The BEVS has become especially popular for small-scale recombinant protein expression in laboratories throughout the world when biologically active proteins are required for research applications. Furthermore, the BEVS is becoming a popular choice for producing commercial farm animal and human vaccines. For example, a subunit/marker vaccine against classical swine fever ("Porcilis Pesti") produced by MSD Animal Health was released in 1998 for vaccinating pigs, a virus-like particle vaccine against cervical cancer ("Cervarix") produced by GlaxoSmithKline was released in 2007 for vaccinating human girls, and an annual trivalent flu vaccine ("Flublok") produced by Protein Sciences was released in 2013 for vaccinating humans.

The third edition of *Baculovirus and Insect Cell Expression Protocols* (the first edition, edited by Christopher D. Richardson, was published in 1995 and the second edition, edited by David W. Murhammer, was published in 2007) was written to provide an updated step-by-step guide to biochemists, molecular biologists, biochemical engineers, and others using the BEVS and/or insect cells for producing recombinant proteins. Furthermore, the third edition of *Baculovirus and Insect Cell Expression Protocols* will provide assistance to scientists

and engineers interested in developing and producing baculovirus insecticides. In both of these cases the procedures involved in producing products at laboratory scale and large scale will be discussed, as well as production in insect larvae.

The third edition of *Baculovirus and Insect Cell Expression Protocols* is divided into seven parts. The first part, entitled "Introduction," contains one chapter that serves as an overview of the major techniques discussed in detail elsewhere in the book. Furthermore, this chapter provides step-by-step procedures involved in quantifying cell growth, baculovirus infection, and cell metabolism. It is strongly recommended that this chapter be read prior to reading your specific chapter(s) of interest. The second part, entitled "Baculovirus molecular biology/development of recombinant baculoviruses," contains three chapters that give an overview of baculovirus molecular biology and methods involved in constructing and isolating recombinant baculoviruses. Moreover, this part contains another chapter that discusses using modified baculoviruses to express genes in mammalian cells ("BacMam"). The third part, entitled "Insect cell culture," contains four chapters that list currently available insect cell lines, methods to isolate new cell lines and develop your own serum-free medium, and routine maintenance and storage of insect cell lines and baculoviruses. The fourth part, entitled "Protein production with recombinant baculoviruses," contains four chapters that discuss small- and large-scale recombinant protein production with the BEVS in both insect and mammalian cell culture and in insect larvae. The other two chapters in this part discuss the large-scale production of virus-like particles and an alternative approach to expressing multicomponent protein complexes. The fifth part, entitled "Recombinant protein production with transformed insect cells," contains a chapter that discusses methods involved in developing stably transformed insect cells for expressing recombinant proteins directly from the insect cell genome, another chapter about improving the protein processing capabilities of host insect cells for use with the BEVS, and a chapter about using *Drosophila* cell lines, which provide an alternative to the lepidopteran insect cell lines used with the BEVS. The sixth part, entitled "Baculovirus development and production for use as insecticides," contains three chapters about the use, production, and characterization of baculoviruses (both wild type and recombinant) for use as biopesticides. The seventh part, entitled "Miscellaneous techniques and applications of the baculovirus/insect cell system," contains three chapters that discuss the use of green fluorescent protein, tubular reactors, and RNAi for research applications. The other two chapters discuss the application of the baculovirus/insect cell system to study apoptosis and generating envelop-modified baculovirus for gene delivery into mammalian cells.

The third edition of *Baculovirus and Insect Cell Expression Protocols* provides the detailed steps required to perform the techniques involved with the use of baculoviruses and insect cell culture and discusses problems that may be encountered. It is hoped that this book will not only aid the user in successfully completing the tasks described herein but also stimulate the development of improved techniques and new applications of baculoviruses and insect cell culture.

The editor would like to thank the 39 contributors for their excellent submissions and the Series Editor, John Walker, for his guidance throughout the process of producing this book. Furthermore, a special thanks to Katie Schnedler for her assistance in converting figures into the proper format.

***Iowa City, IA, USA*** ***David W. Murhammer***

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# Part I

## Introduction

# Chapter 1

# Useful Tips, Widely Used Techniques, and Quantifying Cell Metabolic Behavior

**David W. Murhammer**

## Abstract

The insect cell culture/baculovirus system has three primary applications: (1) recombinant protein synthesis, (2) biopesticide synthesis, and (3) as a model system (e.g., for studying apoptosis). The fundamental techniques involved in these applications are described throughout this book. In this chapter the most widely used techniques are summarized and the reader is directed to detailed information found elsewhere in this book. Furthermore, many useful tips and my personal preferences that are rarely published are discussed in this chapter along with quantitative methods to characterize cell growth, baculovirus infection, and metabolism.

**Key words** Cell growth, Baculovirus infection, Population doubling time, Exponential growth, Cell metabolism, Specific utilization rates, Specific production rates

## 1 Introduction

### 1.1 Cell Growth

The most commonly used lepidopteran insect cell lines are isolates from *Spodoptera frugiperda* (Sf-9 and Sf-21) and *Trichoplusia ni* (Tn-5, which is commonly known as BTI-Tn-5B1-4 and is commercially known as High Five™ cells (Invitrogen); *see* **Note 1**). *See* Chapter 6 for an extensive list of available insect cell lines and Chapter 7 for methods involved in new cell line development. The Sf-9, Sf-21, and Tn-5 cells grow optimally at 27–28 °C and do not require $CO_2$ (due to the buffer system used in the medium). Thus, temperature is the only parameter that needs to be controlled in incubators used to grow these cells.

Following subculture, insect cells in batch culture (*see* **Note 2**) proceed through the typical growth phases [1] (*see* **Note 3** and Fig. 1): (1) lag phase (*see* **Note 4**), (2) exponential growth phase (*see* **Note 5**), (3) stationary phase (*see* **Note 6**), and (4) decline (or death) phase. The lag phase is the time period between subculturing and the exponential growth phase in which the cell growth rate reaches its maximum. Lag phase is usually caused by

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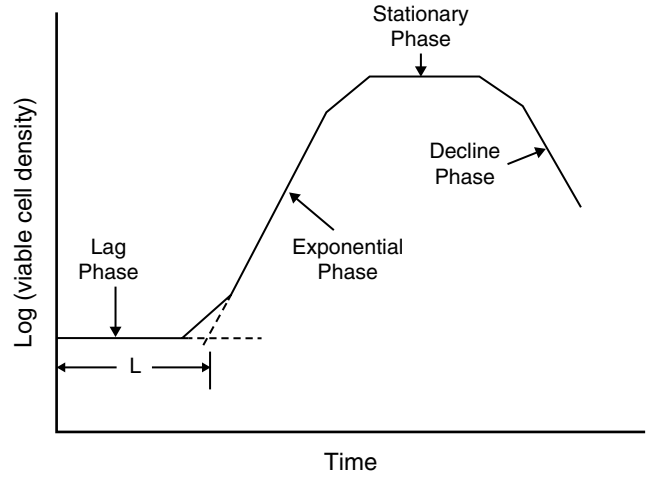


**Fig. 1** A typical cell growth curve (cell density vs. time) showing the lag, exponential, stationary, and decline phases. The length of the lag phase (L) can be found by extending the lag and exponential phase lines and finding the time at which they intersect (as shown)

an environmental change, e.g., using cells from the stationary phase to start a new culture [1] (*see* **Note 7**). During the stationary phase the *net* growth rate is zero, i.e., the cell growth rate is not necessarily zero, but it is equal to the cell death rate. Finally, during the decline phase the cell death rate exceeds the cell growth rate and the viable cell number declines.

The major parameters that are used to characterize cell growth are specific growth rate (μ), population doubling time (PDT), lag time, and maximum cell density. All of these parameters are functions of the specific cell line and growth environment (dissolved oxygen concentration, nutrient concentrations, temperature, pH, etc.). Furthermore, % cell viability is a measure of cell quality. Typical ranges of μ and PDT are 0.029–0.035 $h^{-1}$ and 20–24 h, respectively, for the Sf-9, Sf-21, and Tn-5 cell lines [2]. The lag phase can be eliminated by using cells from the exponential growth phase (*see* **Note 4**). Finally, the maximum cell density in batch culture varies considerably depending upon the cell line and the medium composition, and can exceed $10^7$ cells/mL in some cases [3]. Additional details about these parameters are given under Subheading 3.1.

Cell growth during the exponential growth phase can be represented by

$$N = N_0 \exp(\mu t), \tag{1}$$

where $N$ = cell density (cells/mL) at a given time $t$, $N_0$ = cell density (cells/mL) at time $t = 0$, $\mu$ = specific growth rate ($h^{-1}$), and $t$ = time in culture (h).

### 1.2 Baculovirus Infection

Insect cells are infected with either a recombinant baculovirus (*see* Chapter 4) to produce a recombinant protein or with a wild-type baculovirus to produce biopesticides (recombinant baculovirus can also be used as biopesticides, *see* Chapters 19 and 21). In all of these cases it is critical that a high quality baculovirus (i.e., a pure baculovirus absent of mutants, *see* Subheading 3.2) and healthy cells (i.e., viability > 95 %) in exponential growth be used. The most important parameter involved in baculovirus infection is the multiplicity of infection (MOI), which is the ratio of infectious baculovirus particles to cells. Details about the MOI and other important issues involved in baculovirus infection are given under Subheading 3.2.

### 1.3 Cell Metabolism

Cell metabolism involves characterizing how cells grow, utilize nutrients, and produce products and byproducts. Cell growth, as indicated under Subheadings 1.1 and 3.1, can be characterized by $\mu$, PDT, lag time, and maximum cell density. Other parameters that can be used to characterize cell metabolism are specific utilization (for nutrients) and specific production (for byproducts) rates. For example, we found the specific utilization rates for glucose in uninfected Sf-9 and Tn-5 cells to be $-2.4 \times 10^{-17}$ and $-2.8 \times 10^{-17}$ mol/cell-s (the negative sign in indicative of consumption that results in a reducing the glucose concentration), respectively [2]. Furthermore, it was found that Sf-9 cells did not produce measurable amounts of lactate and ammonium ions when sufficient dissolved oxygen (DO) was present. In contrast, the uninfected Tn-5 cells produced lactate and ammonium ions at rates of $0.7 \times 10^{-17}$ and $5.1 \times 10^{-17}$ mol/cell-s, respectively, when sufficient DO was present. Both the Sf-9 and Tn-5 cells produced alanine (another common byproduct in insect cell culture) at rates of $1.1 \times 10^{-17}$ and $1.5 \times 10^{-17}$ mol/cell-s, respectively. Information about other specific uptake and production rates in these cell lines, both in uninfected and infected cells, can be found in Rhiel et al. [2]. Details about evaluating these parameters are given under Subheading 3.3.

## 2 Materials

### 2.1 Cell Growth

1. Supplies and equipment listed in Chapters 9 and 10 for small-scale cultures or Chapters 11 and 12 for large-scale culture.
2. Coulter particle counter/sizer (Beckman Coulter, Inc., Fullerton, CA) (*see* **Note 8**).

### 2.2 Baculovirus Infection

1. Supplies and equipment listed in Chapter 10 for small-scale cultures or Chapters 11 and 12 for large-scale culture.
2. Coulter particle counter/sizer (Beckman Coulter, Inc., Fullerton, CA) (*see* **Note 8**).

### 2.3 Cell Metabolism

1. Dissolved Oxygen Respirometer (available from Cole Parmer, Vernon Hills, Illinois) (*see* **Note 9**).
2. Nutrient and byproduct monitor, e.g., BioProfile 400 analyzer (Nova Biomedical Corp., Waltham, MA) (*see* **Note 10**).
3. An HPLC for amino acid quantification.

## 3 Methods

### 3.1 Cell Growth

Methods involved in routine cell growth in monolayer (i.e., attached) cultures are given under Subheading 3.1 in Chapter 9. Methods involved in shaker flask culture can be found under Subheading 3.3.5 in Chapter 8 and under Subheading 3.4 in Chapter 10. I prefer the simplicity of using shaker flasks (vs. spinner flasks) for suspension growth experiments where a high level of environmental control is not needed. If a high level of environmental control is necessary, then a bioreactor should be used (e.g., *see* Chapters 11 and 12).

#### 3.1.1 Cell Density and Viability

The cell density (cells/mL) and % viability can be determined with a hemacytometer as indicated under Subheading 3.1.2 of Chapter 11. This involves counting the cells in the four 1 mm × 1 mm squares on the hemacytometer grid as indicated in Fig. 2. This method utilizes the trypan blue dye method for determining cell viability. The

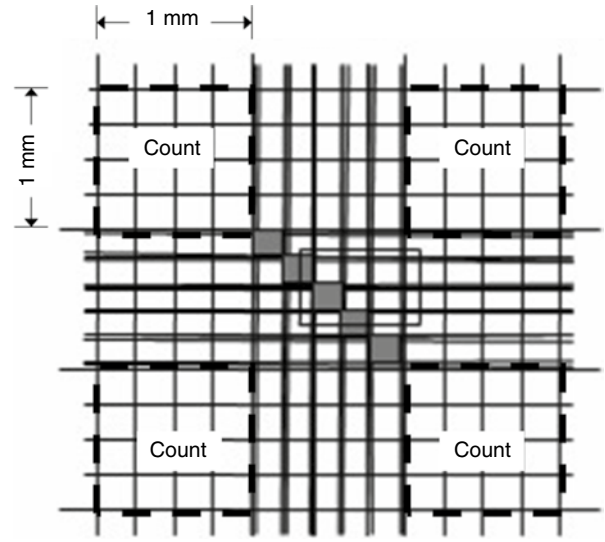


**Fig. 2** Schematic of the hemacytometer grid used to count cells. Typically, the cells in the four corners (designated by "count") are counted. Each of these regions has an area of 1 $mm^2$ and a depth of 0.1 mm; thus, the corresponding volume is 0.1 $mm^3$

underlying principle is that the membranes of the dead cells will be leaky and therefore will readily take up the blue dye and appear blue under the microscope. In contrast, viable (i.e., live) cells have intact membranes that will exclude the dye. While trypan blue dye exclusion is commonly referred to as a measure of cell viability, it is more accurately a measure of membrane integrity. Nonetheless, this is by far the most commonly used method to determine cell viability due to its simplicity and the fact that results can be obtained in ~5 min. The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assay [4] is a more elaborate assay that can be used to provide a more authentic measure of cell viability. The principle behind this assay is that the yellow tetrazolium MTT is reduced by metabolically active cells to produce purple formazan that can be quantified by spectroscopic methods. MTT cell proliferation assay kits are available through many vendors, e.g., the American Type Culture Collection (Manassas, VA; http://www.atcc.org).

I prefer using a Coulter particle counter (*see* **Note 8**) and the trypan blue dye exclusion method for determining the total cell density and cell viability, respectively. (Unfortunately, the Coulter particle counter is a relatively expensive piece of equipment that may be beyond the financial means of some laboratories.) The viable cell density can then be determined by multiplying the total cell density by the fraction of viable cells. Manufacturer instructions can be followed to determine cell densities (and mean cell size) with the Coulter particle counter.

Conducting cell counts in suspension cultures (i.e., spinner flasks, shaker flask, and bioreactors) is much easier than in attached cultures (i.e., tissue culture flasks). Specifically, cell samples can be directly withdrawn from the cultures at the desired times, i.e., every 12–24 h, and continued until the cells are at least in the stationary growth phase (Fig. 1). In contrast, when conducting cell counts for monolayer cultures, the cells must first be detached from the growth surface (*see* Subheading 3.1 in Chapter 9). When obtaining a growth curve for cells in monolayer culture the following procedure should be followed.

1. Equally seed a number of tissue culture flasks equal to the number of time points at which cell counts will be taken (*see* **Note 11**).
2. Detach cells from one tissue culture flask.
3. Determine the cell density (cells/mL) and viability.
4. Repeat **steps 2** and **3** at each time point at which a cell count is to be taken. There should be a sufficient number of flasks seeded to obtain a growth curve into the stationary growth phase (Fig. 1).

**Table 1**
**Cell density versus time data for Sf-9 cell growth in a batch culture**

| Time (h) | Cell density (cells/mL) | Time (h) | Cell density (cells/mL) |
|---|---|---|---|
| 0.0 | $1.02 \times 10^5$ | 112.0 | $1.99 \times 10^6$ |
| 16.5 | $1.06 \times 10^5$ | 136.5 | $3.46 \times 10^6$ |
| 39.0 | $1.67 \times 10^5$ | 159.5 | $4.32 \times 10^6$ |
| 62.5 | $3.05 \times 10^5$ | 192.5 | $4.56 \times 10^6$ |
| 88.0 | $8.37 \times 10^5$ | 208.0 | $4.47 \times 10^6$ |

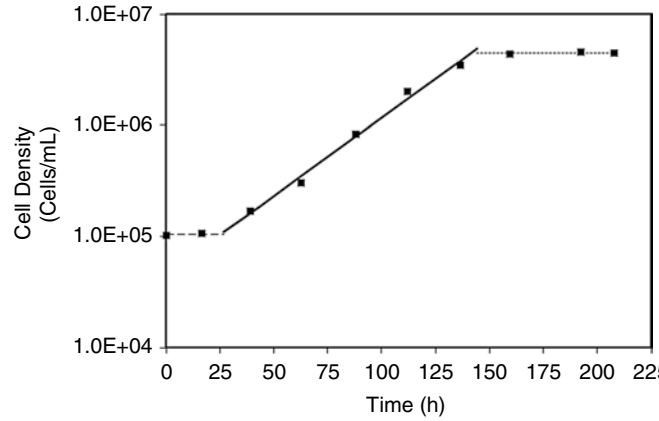


**Fig. 3** Plot of growth curve data (Sf-9 cells grown in serum-free medium) to find the location of the exponential growth phase and the length of the lag phase (*see* Fig. 1)

### *3.1.2 Evaluating μ, PDT, Lag Time, and Maximum Cell Density*

A specific example will be used to demonstrate how these parameters can be determined (Table 1 and Fig. 3).

1. Obtain cell density (cells/mL) versus time data (Subheading 3.1.1). Example data for Sf-9 cell growth is given in Table 1.
2. Plot the data from **step 1** on a semi-log graph (i.e., cell density on the log scale and time on the linear scale) as shown in Fig. 3 for our example.
3. Find the time range over which exponential phase occurs, i.e., where the data are approximately linear on the semilog plot. For our example shown in Fig. 3, the exponential phase data occur from 39.0 to 136.5 h.
4. Calculate the natural logarithm (ln) of the exponential phase cell densities (between 39.0 and 136.5 h for our example).

5. Plot the ln(cell density) ($y$-axis) versus time ($x$-axis) on a linear plot and determine the best linear fit. The slope of this plot is the specific growth rate ($\mu$), as can be seen by rearranging Eq. 1:

$$\ln(N) = \ln(N_0) + \mu t. \tag{2}$$

   For our example (Table 1 and Fig. 3) the corresponding slope is 0.0325; thus, $\mu = 0.0325\ h^{-1}$.

6. Calculate the population doubling time (PDT) as follows:

$$\mathrm{PDT} = \frac{\ln(2)}{\mu}. \tag{3}$$

   For our example, $\mathrm{PDT} = \ln(2)/(0.0325\ h^{-1}) = 21.3$ h.

7. Determine the length of the lag phase by finding the intersection between the extension of the lag phase and exponential phase as illustrated in Fig. 1. For our example (*see* **Note 12**), the length of the lag phase ~27 h (Fig. 3).

8. Determine the maximum cell density. For our example, the maximum cell density is $4.56 \times 10^6$ cells/mL (Table 1).

### 3.1.3 Cell Growth: Useful Tips

Some of these tips were discussed within the text above, but are repeated here for easy reference and to emphasize their importance.

1. Rinse glassware (e.g., Erlenmeyer flasks used as shaker flasks) used in cell culture very well to remove all traces of detergent. We have found that very low detergent concentrations can inhibit cell growth. I recommend that the use of detergents be minimized if not totally eliminated.

2. I prefer using shaker flasks instead of spinner flasks to grow and infect insect cells for a number of reasons. First, incubator shakers commonly used for bacterial work can be used. The one requirement is that temperature can be controlled at 27–28 °C (cells will grow at lower temperatures, e.g., 23–25 °C, but at a significantly slower rate). Second, shaker flasks are more economical, i.e., they are simply Erlenmeyer flasks. Adding to the expense of spinner flasks, I have found that spinner flask parts need to be replaced periodically as autoclaving seems to accelerate their wear out rate. On the other hand, the shaker flasks do not require maintenance and will last until broken. Third, the flow pattern is more consistent in the shaker flasks. I have found that agitation becomes "jerky" in many spinner flask designs after being autoclaved a few times.

3. Use "conditioned" medium (i.e., cell-free medium isolated from mid-exponential phase cultures) when cell cloning. We have found that individual cells will not grow in fresh medium. Apparently, the cells produce a substance(s) that the cells

require to divide and this substance(s) is present in the "conditioned" medium.

4. Be aware of potential nutrient deprivation (especially glucose and glutamine), dissolved oxygen limitations and/or toxic byproduct accumulation (especially lactate and ammonium ions) in cell cultures, especially at high cell densities.
5. Heat inactivation of fetal bovine serum (FBS) may not be necessary (*see* **Note 4** in Chapter 16). There seems to be some controversy over this issue; therefore, I suggest that you compare cell growth with and without heat inactivation if you use FBS.
6. Grow the cells in the dark (this may require, e.g., covering a glass bioreactor with an opaque material; we use aluminum foil). To my knowledge no formal study has investigated this issue, but it is believed that light can break down medium components into toxic products.
7. Insect cell lines are generally heterogeneous populations (even those that were originally cloned) as cell properties change upon passage in cell culture. Therefore, cloning should be performed if a homogeneous cell population is desired. Furthermore, I recommend freezing many vials of your cell culture that can be thawed periodically for consistent cell behavior in a series of experiments (*see* Subheadings 3.3.8 and 3.3.9 in Chapter 8 and Subheadings 3.3.3 and 3.3.4 in Chapter 9).
8. Insect cells are not as shear sensitive as commonly reported. E.g., I have found that increased agitation rates in bioreactors do not result in cell damage in the absence of bubble incorporation through cavitation, etc. (*see* **Note 5** in Chapter 14). Note that a surfactant (usually Pluronic® F-68) is commonly added to cell cultures to protect cells from bubble damage.
9. Do not allow cells to overgrow (i.e., spend minimal time in stationary phase; it is best to maintain cells in exponential growth phase). I have found that cell properties can change as cells are allowed to remain in stationary phase for an extended time. I recommend that cells be subcultured in late exponential growth phase prior to entering the stationary phase.
10. Seed cultures at a sufficient cell density. Cells will go through a lag phase if seeded at too low a density (especially in serum-free medium). I recommend $5 \times 10^5$ cells/mL as a reasonable target seeding density.
11. Do not use antibiotics in routine cell culture (*see* Subheading 3.2.1 in Chapter 9). Use of antibiotics can mask low levels of microorganisms in the cell culture whose presence would quickly become obvious in the absence of antibiotics.

## 3.2 Baculovirus Infection

### 3.2.1 Recombinant Baculovirus Development

The first step in using the BEVS for producing a recombinant protein is to construct the recombinant baculovirus (*see* Chapters 3 and 4 for details). If a pure recombinant protein is desired, then it is recommended that a baculovirus construct be made that adds a polyhistidine tail to the protein. The resulting polyhistidine tag has a strong affinity for binding transition metals. This property can be used to remove the polyhistidine-containing protein from a mixture of proteins in solution. The polyhistidine tag can then be enzymatically removed to obtain the purified protein of interest. Kits are commercially available for purifying these polyhistidine-containing recombinant proteins, e.g., the Ni-NTA Purification System available from Life Technologies (Grand Island, New York).

It is critical that the recombinant baculovirus be pure prior to its amplification and use in producing the recombinant protein. Therefore, the baculovirus should be plaque purified as described under Subheading 3.3 of Chapter 4.

### 3.2.2 Baculovirus Amplification

It is recommended that the baculovirus (Subheading 3.2.1) be amplified in shaker flasks or bioreactors (depending on the amount of baculovirus stock required). It is critical to use either Sf-9 or Sf-21 cells for producing baculovirus stocks (Tn-5, while an excellent cell line for producing recombinant proteins, is not well suited for producing baculovirus stocks) and that these cells are infected at a relatively low MOI (~0.1) to prevent mutant accumulation (Subheading 3.4.1 in Chapter 9). Furthermore, it has been found that the addition of fetal bovine serum (5–10 %) significantly enhances the stability of baculovirus stocks stored at 4 °C. Under these conditions the baculovirus is stable for up to ~1 year. Alternatively, baculovirus stocks can be stored at −85 °C for long periods (*see* Subheading 3.4.2 in Chapter 9).

### 3.2.3 Determining Baculovirus Titer

It is critical that an accurate (*see* **Note 13**) baculovirus titer (plaque forming units/mL, PFU/mL; effectively the concentration of infectious baculovirus particles) is known when infecting insect cells. The most commonly used methods to determine baculovirus titer are the plaque assay and end point dilution (also referred to as limiting dilution). Both of these methods directly measure baculovirus infectivity in cells and therefore provide a measure of infectious baculovirus concentration. The plaque assay procedure is described under Subheading 3.5 in Chapter 4. The basic end point dilution procedure is described under Subheading 3.4 in Chapter 10. A critical step in this procedure is identifying positive wells (i.e., wells that contain cells infected by a baculovirus). The procedure given in Chapter 10 relies on direct identification of positive wells (which can be difficult for recombinant baculoviruses lacking the polyhedrin gene). Alternative procedures for identifying positive wells include the use of the anti-gp64 antibody (Subheading 3.4 in Chapter 5) and the green fluorescent protein

(GFP) (Subheading 3.2 in Chapter 22). The anti-gp64 antibody procedure is based on the principle that the gp64 protein is expressed on the surface of baculovirus infected cells. The GFP procedure is based on detecting GFP (this method requires inserting the GFP gene into the baculovirus genome as described under Subheading 3.1 in Chapter 22) following baculovirus infection. Another method of tittering baculovirus is through flow cytometry following binding of a fluorescent dye to baculovirus DNA (*see* **Note 20** in Chapter 11). This procedure provides a total baculovirus concentration in contrast to the plaque assay and end point dilution procedures that provide the desired infectious baculovirus concentration.

The baculovirus tittering methods described above can best be verified by infecting cell cultures at a range of concentrations and observing cell growth or the lack thereof (*see* Subheadings 3.2.4 and 3.2.6).

### 3.2.4 Poisson Distribution

The Poisson distribution [5] expresses the probability of a number of events occurring during a given time period and can be used to model the baculovirus infection process. First, the fraction of cells infected by a specific number of baculovirus particles at a given MOI can be predicted from

$$F(n,\mathrm{MOI}) = \frac{(\mathrm{MOI})^n \exp(-\mathrm{MOI})}{n!}, \tag{4}$$

where $F(n, \mathrm{MOI})$ is the fraction of cells infected with n baculovirus particles when a multiplicity of infection of MOI is used.

The fraction of baculovirus infected cells can be predicted by subtracting the fraction of uninfected cells (i.e., using $n = 0$ in Eq. 4) from 1.0:

$$\begin{aligned}&(\text{Fraction of insect cells infected with Baculovirus at a given MOI})\\ &\quad = 1 - \exp(-\mathrm{MOI}).\end{aligned} \tag{5}$$

### 3.2.5 Infection of Insect Cell Cultures: Cell Line Selection

Some issues to consider when infecting an insect cell culture include (1) the cell line to use, (2) the cell density at which the cells should be infected, (3) the MOI to use, and (4) the product harvest time.

Sf-9 and Tn-5 are the most commonly used host cell lines used to express recombinant proteins with the BEVS. It has been demonstrated that the Tn-5 cell line expresses higher levels of recombinant proteins in many, but not all, cases [6] and therefore is usually a good choice for producing recombinant proteins. The Mimic™ Sf-9 cell line commercially available from Life Technologies (*see* **Note 14**) is a good choice if the recombinant protein is glycosylated. The reader is also referred to Chapter 6 regarding the wide range of available insect cell lines and Chapter 7 regarding methods to develop new cell lines, as well as Chapter 18

regarding cell line modification to improve protein processing. Finally, Chapter 8 can be consulted regarding selecting an appropriate serum-free medium (*see* **Note 15**).

### 3.2.6 Infection of Insect Cell Cultures: Time of Infection and MOI

The time of infection (i.e., the cell density at infection) and the MOI are interrelated due to such competing factors as the effect of MOI on cell growth (*see* **Note 16**) and the potential for nutrient depletion (*see* **Note 2**). In small-scale cultures it is almost always desirable to use an MOI high enough to result in a synchronous infection. I recommend using an MOI of 10 and infecting the cells at a density of $1.5–2.0 \times 10^6$ cells/mL. It is critical that the cells be healthy (viability > 95 %) and be in exponential growth phase. Note that the Poisson distribution (Eq. 5) predicts that 99.995 % of the cells are infected by the primary infection when an MOI of 10 is used. Therefore, cell density should not increase following infection at an MOI of 10. If the cell density does increase, then the titer of the baculovirus stock has likely been overestimated.

In contrast, in large-scale culture it might be desirable to infect at a low MOI to minimize the volume of required baculovirus stock that needs to be added. In this case, the uninfected cells will continue to grow until all of the cells in culture have been infected. The fraction of cells infected by the primary infection can be predicted by the Poisson distribution (Eq. 5). For example, the Poisson distribution predicts that infecting cells at MOIs of 0.1 and 1.0 would result in approximately 9.5 % and 63.2 % of the cells being infected by the primary infection, respectively. It is possible that nutrients may become depleted (dependent upon the specific infection strategy used) and therefore nutrient addition (i.e., a fed-batch approach) may be desirable if a significant amount of cell growth occurs following baculovirus addition due to using low MOIs.

The procedures used to infect insect cell cultures to produce recombinant proteins are given in Chapters 10 and 11 for small-scale and large-scale production, respectively.

### 3.2.7 Infection of Insect Cell Cultures: Product Harvest Time

It is critical that the recombinant protein product be harvested before degradation by proteases that are either secreted by cells or released by cells lyzed by the baculovirus infection (this is of particular importance for secreted proteins). A general guideline is to harvest the recombinant protein when the cell viability decreases to 75–80 %, although this should be adapted as appropriate to each specific case.

### 3.2.8 Baculovirus Infection: Useful Tips

Some of these tips were discussed within the text above, but are repeated here for easy reference and to emphasize their importance.

1. Baculoviruses should be produced in Sf-9 or Sf-21 cell cultures; Tn-5 cells should not be used for this purpose.

2. Use a low MOI (~0.1) when producing baculovirus stocks to prevent mutant accumulation (Subheading 3.4.1 in Chapter 9).
3. The stability of baculoviruses stored at 4 °C can be increased significantly by adding 5–10 % fetal bovine serum (FBS). The addition of FBS to the insect cell cultures during the infection process, however, could complicate subsequent recombinant protein purification due to the high concentration of proteins in FBS. Alternatively, the baculovirus stocks can be stably stored at −85 °C for long term (Subheading 3.4.2 in Chapter 9).
4. Cells stop dividing upon baculovirus infection. Therefore, increased cell density, or the lack thereof, following baculovirus infection can be used to estimate the "true" titer of a baculovirus stock.
5. Baculovirus stock titers are usually determined in Sf-9 or Sf-21 cells lines. It is important to note, however, that the effective titer in Tn-5 cells will be significantly higher (apparently, baculovirus uptake by the Tn-5 cell line is much more efficient). Thus, if a baculovirus stock titered in Sf-9 or Sf-21 cells is to be used to infect Tn-5 cells, then it would be useful to test various concentrations of the baculovirus stock in Tn-5 cell culture (*see* Subheading 3.2.4) to obtain a "true" titer of a baculovirus stock for Tn-5 cell culture.
6. Small-scale insect cell cultures can be gently centrifuged (we use either $400 \times g$ for 10 min or $1000 \times g$ for 5 min) 1–4 h following baculovirus infection (I recommend 4 h to provide sufficient time for complete baculovirus uptake by the cells; some researchers believe that 1 h is sufficient) and resuspended in fresh medium to assure that nutrients are not depleted. Care must be taken in the resuspension step as the cells can easily be damaged. It should be realized that this step will take time to perform, i.e., one should not try to do this rapidly by, e.g., vortexing, or the cells will certainly be damaged. I recently had a student who seemed to be incapable of resuspending cells without damaging them and therefore had him eliminate the resuspension step. In this particular case the cell cultures did not suffer from nutrient depletion. Note that the likelihood of nutrient depletion is dependent upon the specific medium used (most commercial serum-free medium have relatively high nutrient levels) and the baculovirus infection protocol used (i.e., combination of MOI and cell density at infection, noting that more nutrients will be used at higher cell densities and if the cells grow following the initial baculovirus infection). In large-scale cultures, additional nutrients can be added following baculovirus infection if deemed necessary (i.e., a fed-batch culture can be used). Obviously, this approach could also be used in small-scale cultures. In either case, however, it is critical that the content of the nutrient addition be consistent

with the cellular needs, which can be determined following procedures outlined under Subheading 3.3.

7. It is tempting simply to use media with very high levels of nutrients in order to prevent nutrient depletion. This approach, however, does not seem to work. For example, increasing the initial glucose concentration beyond a certain level (I cannot find any definitive evidence regarding the specific concentration) does not result in increased maximum cell density prior to glucose depletion. I suspect that this is due to an increased glucose consumption rate; furthermore, very high glucose concentrations may actually be inhibitory. It is certainly no accident that most commercial insect cell culture media contain maltose (a glucose dimer) in addition to glucose. The maltose is slowly broken down into glucose as the cells grow, thereby resulting in a relatively constant glucose concentration in the medium until all of the maltose is depleted. I suspect (although I cannot find specific evidence in support) that the insect cells release an enzyme into the medium that breaks down the maltose. Finally, it is important to account for the presence of maltose in the medium when quantifying glucose utilization rates (Subheading 3.3).

## 3.3 Cell Metabolism

Quantifying cell metabolism is useful for both characterizing cell behavior (e.g., lactate accumulation is usually indicative of inefficient use of glucose and for some cell lines, e.g., Sf-9, is indicative of oxygen deficiency) and for designing media and developing nutrient feeding strategies. Specific utilization and production rates are used to quantify nutrient consumption and byproduct production, respectively.

### 3.3.1 Specific Oxygen Utilization Rate: Using a Dissolved Oxygen (DO) Electrode

1. If a dissolved oxygen electrode is being used (e.g., in a bioreactor), then turn off the oxygen supply and record the dissolved oxygen (DO) concentration as a function of time. Do not allow the DO concentration to fall below 20 % air saturation (*see* **Notes 17–19**).
2. Plot the DO concentration ($y$-axis) versus time ($x$-axis) on linear axes.
3. Find the slope of the linear portion of the curve obtained in **step 2**. The resulting slope represents the volumetric oxygen utilization rate (OUR).
4. Determine the viable cell density (cells/mL) as described under Subheading 3.1.1.
5. Calculate the specific OUR by dividing the volumetric OUR obtained in **step 3** by the viable cell density obtained in **step 4**. This step requires that the DO concentration in terms of % air saturation be converted to DO concentration in terms of moles/L (M). The solubility of oxygen in pure water when air

is in the gas phase at 27 °C is ~0.26 mM [7]. Thus, 100 % air saturation corresponds to ~0.26 mM oxygen. If desired, corrections can be made for the effect of salts (i.e., actual medium vs. pure water) in the solution on oxygen solubility [8]; however, the correction is usually small and can be ignored for most applications.

6. Example: assume that we find a slope, i.e., a volumetric OUR, of –2.8 % DO/min from **step 3** at a cell density of $2.5 \times 10^6$ cell/mL; the specific OUR can then be calculated:

$$(\text{Specific OUR}) = \frac{\left(-2.8\dfrac{\%\text{DO}}{\text{min}}\right)\left(\dfrac{\text{min}}{60\,\text{s}}\right)\left(\dfrac{0.26\,\text{mM}}{100\%\text{DO}}\right)\left(\dfrac{\text{mol}/\text{L}}{10^3\,\text{mM}}\right)}{\left(2.5\times10^6\,\text{cells}/\text{mL}\right)\left(1000\,\text{mL}/\text{L}\right)}$$
$$= -4.9\times10^{-17}\,\text{mol}/\text{cell-s}$$

*3.3.2 Specific Oxygen Utilization Rate: Using a Dissolved Oxygen (DO) Monitor*

1. If a DO electrode is not present, then a DO monitor can be used.
2. Remove the appropriate volume of cell suspension from the culture (appropriate for the DO monitor chamber being used) and place in the DO monitor chamber.
3. Record the DO concentration as a function of time. Note that the DO concentration can be monitored to lower DO concentrations than in cell culture (Subheading 3.3.1) since the cells will be discarded when the test is completed and it does not matter if the cell metabolism is affected.
4. Follow **steps 2–5** given under Subheading 3.3.1 to determine the specific OUR.

*3.3.3 Specific Nutrient Utilization and Byproduct Accumulation Rates*

As shown below, the methods used to calculate the specific nutrient utilization and byproduct accumulation rates are different during exponential growth than when the cell density is constant (i.e., in stationary growth phase and in baculovirus infected cultures).

*3.3.4 Specific Rates During Exponential Growth Phase*

1. Determine the viable cell density (Subheading 3.1.1) and nutrient (e.g., glucose) and/or byproduct (e.g., lactate ion) concentration as a function of time in culture (*see* **Note 20**).
2. Determine the range of times over which cells are in the exponential phase.
3. Determine the specific growth rate (Subheading 3.1.2).
4. Plot cell density (*y*-axis) versus component of interest concentration (*x*-axis) using exponential phase data only and determine the slope. For a nutrient this slope is referred to cell yield coefficient.
5. The specific nutrient utilization rate (or specific byproduct production rate) is then evaluated by dividing the specific

growth rate (obtained in **step 3**) by the slope found in **step 4**. Note that a utilization rate will be negative (i.e., nutrient concentration decreases with time) and a production rate will be positive (i.e., byproduct concentration increases with time).

6. Example: Consider the data for Sf-9 cell culture given in Table 2.
   (a) Plotting ln(cell density) versus time yields a specific growth rate ($\mu$) of 0.0313 $h^{-1}$ for the exponential phase (−72 to 0 h pi). This follows the procedure given under Subheading 3.1.2 and Fig. 3.
   (b) Plot the cell density versus glucose concentration data for the exponential growth data (for times from −72 to 0 h pi) as shown in Fig. 4. For our example the slope is $-1.16\times10^5$ cells/(mL-mM).

**Table 2**
**Cell density and glucose concentration as a function of time in Sf-9 cell culture. Note that the cells were allowed to grow 72 h prior to baculovirus infection at an MOI of 10**

| Time post-infection (h) | Viable cell density (cells/mL) | Glucose concentration (mM) |
|---|---|---|
| −72 | $2.2\times10^5$ | 48.0 |
| −48 | $4.8\times10^5$ | 45.5 |
| −24 | $1.0\times10^6$ | 39.5 |
| 0 | $2.1\times10^6$ | 32.0 |
| 24 | $2.3\times10^6$ | 21.5 |
| 48 | $2.1\times10^6$ | 11.5 |
| 72 | $2.2\times10^6$ | 5.5 |

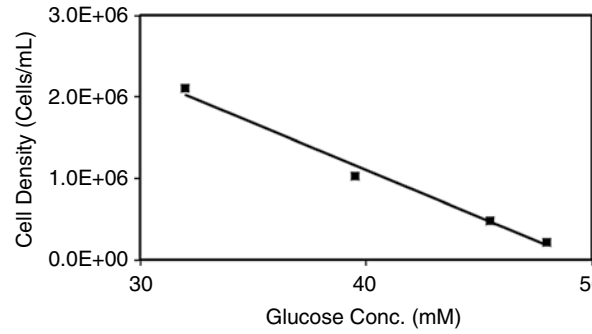


**Fig. 4** Plot of cell density versus glucose concentration in the exponential phase for Sf-9 cell culture in serum-free medium. The slope of this curve is used to determine the specific glucose consumption rate during exponential phase

(c) Calculate the specific utilization rate of glucose as follows:

$$\text{(Specific glucose utilization rate)} = \frac{\left(0.0313\text{h}^{-1}\right)\left(\frac{\text{h}}{3600\text{s}}\right)}{\left(-1.16\times10^{5}\,\frac{\text{cells}\,/\,\text{mL}}{\text{mM}}\right)\left(10^{3}\,\frac{\text{mM}}{\text{mol}\,/\,\text{L}}\right)(1000\text{mL}\,/\,\text{L})}$$
$$= -7.5\times10^{-17}\,\text{mol}\,/\,\text{cell-s}$$

### 3.3.5 Specific Rates During Constant Cell Density

This approach should be used for cells in the stationary phase or in a baculovirus-infected culture in which all the cells have been infected, i.e., when the cell density is constant.

1. Determine the cell density (Subheading 3.1.1).
2. Determine the component concentration at many different time points (I recommend at least 3–4 data points with at least 6–8 h between them) (*see* **Note 20**).
3. Plot the component concentration (*y*-axis) versus time and determine the slope.
4. Calculate the specific utilization rate (or specific production rate for a byproduct) by dividing the slope determined in **step 2** by the cell density.
5. Example: consider the baculovirus infected cell data given in Table 2 for times ranging from 0 to 72 h pi. Note that this is an example where the cell density is essentially constant. For our specific example the mean cell density for the 4 postinfection data points is $2.2\times10^{6}$ cells/mL.
   (a) Plot the glucose concentration versus time as given in Fig. 5. For our example the slope is –0.373 mM/h.

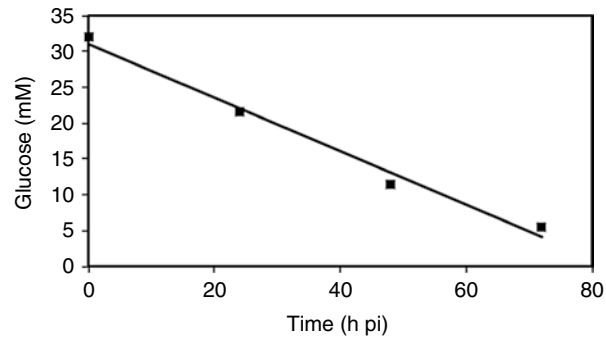


**Fig. 5** Plot of glucose concentration versus time following baculovirus infection (MOI = 10) of Sf-9 cell culture in serum-free medium at a constant cell density of ~$2.2\times10^{6}$ cells/mL. The slope of this curve is used to determine the specific glucose consumption rate following a synchronous baculovirus infection

(b) Calculate the specific glucose utilization rate as follows:

$$\text{(Specific glucose utilization rate)} = \frac{\left(-0.373\frac{\text{mM}}{\text{h}}\right)\left(\frac{\text{h}}{3600\,\text{s}}\right)\left(\frac{\text{mol}/\text{L}}{10^3\,\text{mM}}\right)}{\left(2.2\times10^6\,\text{cells}/\text{mL}\right)\left(1000\,\text{mL}/\text{L}\right)}$$
$$= -4.7\times10^{-17}\,\text{mol}/\text{cell-s}$$

## 4 Notes

1. The nomenclature used in naming some cell lines contains the laboratory in which the cell line was first isolated. For example, the BTI-Tn-5B1-4 cell line was isolated at the Boyce Thompson Institute ("BTI") of Cornell University and the IPLB-Sf21-AE (full name of the Sf-21 cell line) was isolated at what was then called the Invertebrate Pathology Laboratory Beltsville ("IPLB").
2. The most common culturing method is batch, which simply consists of adding cells to fresh medium and allowing the cells to grow as shown in Figs. 1 and 3. Alternative culturing methods include fed-batch and perfusion. Fed-batch culture begins as a batch culture, but nutrients are added periodically to overcome nutrient depletion. Thus, the maximum cell density obtained in fed-batch cultures can be significantly higher than those obtained in batch culture. Perfusion culture involves continually flowing fresh medium through a system in which the cells are retained. Thus, perfusion culture replenishes nutrients *and* removes potentially inhibitory toxic byproducts and therefore provides an excellent model of the in vivo environment.
3. While I prefer the four growth phases shown in Fig. 1, other sources (e.g., ref. 1) include two additional growth phases, i.e., accelerating growth phase between the lag and exponential growth phases and decelerating growth phase between the exponential and stationary growth phases.
4. The length of the lag phase can be minimized by subculturing with cells from mid-exponential growth phase. I have found essentially no lag phase with Sf-9 and Tn-5 cells when cells from mid-exponential growth phase are used to seed new cultures.
5. The exponential growth phase is commonly referred to as logarithmic growth. I prefer the term "exponential growth" since it is a more accurate representation of actual cell behavior.
6. The length of the stationary phase can vary dramatically between cell lines. For example, I have found that the length of the stationary phase is usually much longer for Sf-9 cell growth than for Tn-5 cell growth.

7. Cells usually enter the stationary phase as a result of running out of a critical nutrient(s) (e.g., glucose and/or glutamine) and/or accumulation of toxic byproduct(s) (e.g., lactate and/or ammonium ions). Furthermore, protein synthesis (some of which are necessary for cell division) in stationary phase cells is generally reduced significantly compared to exponential phase cells. Therefore, stationary phase cells used to seed a new culture will have to adapt to a completely different environment (e.g., increased nutrient concentrations) and commence protein synthesis.
8. Purchase of a Coulter particle counter/sizer is optional since cell counts can also be obtained through the use of a hemacytometer. However, I prefer using the particle counter to obtain total cell counts (note that this instruments cannot distinguish between viable and nonviable cells) and the trypan blue exclusion method to determine cell "viability." (Note that the trypan blue exclusion method is actually a measure of membrane integrity, but is commonly used as a measure of cell viability due to its ease of use.) I have found the particle counter to be more accurate and this instrument also provides the cell size distribution. The cell size distribution is particularly useful in monitoring baculovirus infection, i.e., cell size increases as the baculovirus infection advances.
9. This oxygen monitor is applicable for small-scale cultures, e.g., shaker flasks. If a dissolved oxygen electrode is being used (e.g., in a bioreactor), then the electrode should be used as described under Subheading 3.3.1.
10. This bioprocess monitor will quantify glucose, lactate, glutamine, glutamate, ammonium, dissolved oxygen, and carbon dioxide. Furthermore, it will determine the culture pH.
11. It is critical that a given T-flask is only used for cell counts at one time point since cell growth is significantly disrupted by the cell detachment process.
12. In our example the long lag phase was probably due to seeding at too low of a cell density (~$10^5$ cells/mL, *see* Table 1) in serum-free medium. I would recommend that a seeding density of at least $5 \times 10^5$ cells/mL be used with serum-free medium.
13. Note that the accuracy of the baculovirus titers should be viewed from the perspective that the assays are performed on a log scale (i.e., tenfold dilutions are used) and therefore the errors on a linear scale may be large. I believe that errors as large as 40–50 % on a linear scale are not unexpected. Thus, if a higher level of accuracy is desired, then cells should be infected at a range of baculovirus concentrations and the titer estimated by determining how much cells grow following infection through the use of the Poisson distribution (Subheading 3.2.4).

14. The Mimic™ Sf-9 cell line is the same as the SfSWT-1 cell line developed by the Donald L. Jarvis laboratory. This cell line contains the following mammalian glycosyltransferase genes: α2,6-sialyltransferase, α2,3-sialyltransferase, β4-galactosyl transferase, *N*-acetylglucosaminyltransferase I, and *N*-acetylglucosaminyltransferase II. Thus, this cell line has the capability to produce recombinant proteins with terminally sialylated *N*-glycans that cannot be produced in wild-type Sf-9 cells. *See* Chapter 18 regarding strategies utilized to produce this and other modified cell lines that can be used to improve protein processing.
15. Serum-free medium should be used to produce recombinant proteins, especially if the protein is secreted, since serum contains large amounts of proteins that increases the difficulty of obtaining the pure recombinant protein. Furthermore, a baculovirus vector that adds a polyhistidine tag should be used to simplify purification (Subheading 3.2.1).
16. When an insect cell is infected with a baculovirus it stops dividing. Therefore, infecting an insect cell culture at a high MOI will result in infecting all of the cells in the culture (i.e., a synchronous infection) and cell growth will cease (i.e., the cell density will remain constant until cell death causes a cell density reduction). Conversely, infecting an insect cell culture at a low MOI will result in many uninfected cells that will continue to divide until they are infected by a secondary infection (i.e., these uninfected cells will be infected by baculoviruses produced by the cells infected by the primary infection); thus, the overall cell density will increase. These extreme examples illustrate how the trend in cell density following baculovirus infection and the Poisson distribution (Eq. 5) can be utilized to estimate the titer in the baculovirus solution.
17. 100 % air saturation is the concentration of dissolved oxygen in solution when the solution is in equilibrium with an air (which contains 21 mole % oxygen) gas phase, i.e., it is the solubility of oxygen in the aqueous solution under these conditions. Note that using a pure oxygen gas phase can increase the oxygen solubility by a factor of ~5 (actually 100/21).
18. It is strongly recommended that the dissolved oxygen concentration not be allowed to go below 20 % air saturation when measuring the oxygen utilization rate after turning off the oxygen supply as this could alter cell metabolism.
19. Determining the oxygen utilization rate by monitoring the dissolved oxygen concentration after turning off the oxygen supply is dependent upon only oxygen within the cell culture being utilized. Therefore, one must either account for oxygen being transferred from the headspace (generally not an easy task) or eliminate oxygen in the head space (e.g., by purging with nitrogen). I recommend the latter approach.

20. Many of the nutrients and byproducts can be determined using a bioprofile analyzer by following the manufacturer directions. Furthermore, an HPLC can be used to determine amino acid concentrations as described in reference [2].

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# Part II

## Baculovirus Molecular Biology/Development of Recombinant Baculoviruses

# Chapter 2

# Introduction to Baculovirus Molecular Biology

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## Abstract

The development of baculovirus expression vector systems has accompanied a rapid expansion of our knowledge about the genes, their function and regulation in insect cells. Classification of these viruses has also been refined as we learn more about differences in gene content between isolates, how this affects virus structure and their replication in insect larvae. Baculovirus gene expression occurs in an ordered cascade, regulated by early, late and very late gene promoters. There is now a detailed knowledge of these promoter elements and how they interact first with host cell-encoded RNA polymerases and later with virus-encoded enzymes. The composition of this virus RNA polymerase is known. The virus replication process culminates in the very high level expression of both polyhedrin and p10 gene products in the latter stages of infection. It has also been realized that the insect host cell has innate defenses against baculoviruses in the form of an apoptotic response to virus invasion. Baculoviruses counter this by encoding apoptotic-suppressors, which also appear to have a role in determining the host range of the virus. Also of importance to our understanding of baculovirus expression systems is how the virus can accumulate mutations within genes that affect recombinant protein yield in cell culture. The summary in this chapter is not exhaustive, but should provide a good preparation to those wishing to use this highly successful gene expression system.

**Key words** Baculovirus, Gene expression, Promoters, Insect cells, Virus structure, Virus replication, Apoptosis

## 1 Introduction

The last 20 years have seen baculoviruses maintain a reputation for producing high yields of recombinant proteins in insect cells. Despite the perceived difficulties of working with a virus in eukaryotic cells, the fact that posttranslational modifications such as glycosylation, fatty acid acylation, disulphide bond formation, and phosphorylation are carried out very similar to the same processes in mammalian cells has convinced many users of the value of baculoviruses as expression vectors. Continuous development of the system by many groups has seen the early problems of making recombinant viruses a distant memory. Using baculoviruses as expression vectors is no longer the preserve of specialist virologists.

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The many commercial kits now available have brought the system within the capabilities of any competent molecular biologist. Most of these kits are based on the prototype member of the Baculoviridae, namely *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and utilize the highly expressed polyhedrin gene promoter. The simplicity of culturing insect cells such as *Spodoptera frugiperda* or *Trichoplusia ni* makes scale up of recombinant protein production feasible for most laboratories. The problems experienced with spinner or suspension cultures where shear forces limited viability have largely been solved with the introduction of serum-free media allied with antifoam and protective (e.g., Pluronic® F-68) agents. The exploitation of baculoviruses as biosafe insecticides has also benefited from the work on expression vectors and fundamental studies on virus gene function. While most baculovirus expression vectors lack the original polyhedrin gene required for making occluded viruses, recombinant virus insecticides can preserve this process by utilizing nonessential regions of the virus genome for the insertion of foreign genes encoding insecticidal proteins.

## 2 Classification

The Baculoviridae are a family of DNA viruses with circular double stranded genomes that only infect arthropods. They are characterized by their ability to occlude virions in a crystalline protein matrix to form either polyhedra or granules. Viruses forming polyhedra are known as nucleopolyhedroviruses (NPVs) and those forming granules as granuloviruses. This subdivision is based on a number of criteria, including occlusion body morphology and the mechanism by which nucleocapsids are enveloped in infected cells [1]. Granuloviruses produce small occlusion bodies (OBs) ($0.16-0.30\ \mu m \times 0.30-0.50\ \mu m$) normally containing one or occasionally two virions encapsulated in a protein called granulin. Nucleopolyhedroviruses produce larger occlusion bodies (0.5–1.5 μm in diameter) composed of polyhedrin protein, which contain many virions. The family is divided into four genera that only infect arthropods. The Alphabaculoviruses and Betabaculoviruses comprise nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) that are isolated from Lepidoptera (butterflies and moths). Figure 1 illustrates an example of each of these genera. Deltabaculoviruses and Gammabaculoviruses comprise NPVs and infect dipteran (flies) and hymenopteran (sawflies) species, respectively. The NPVs pathogenic for members of the order Lepidoptera have been further subdivided into groups I and II based on molecular phylogenies [1, 2]. An occluded virus also infects the pink shrimp *Penaeus monodon* [3–5]. Partial sequence analysis showed that it encodes proteins similar to those produced by baculoviruses.

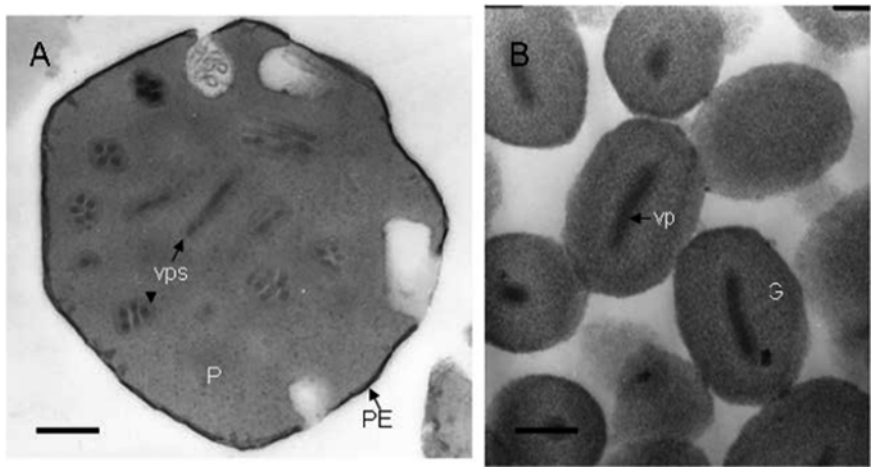


**Fig. 1** Electronmicrographs of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) polyhedron (**a**) and *Plodia interpunctella* granulovirus (PiGV) granules (**b**). Note the multiple virus particles (vps) in longitudinal or cross section in AcMNPV, whereas in PiGV each granule contains a single virus particle (vp). Virus particles are occluded within polyhedrin protein (P) or granulin protein (G). Polyhedra are bounded by an envelope (PE), which contains protein and carbohydrate. Scale bar is 200 nM

## 3 Baculovirus Structure

The double stranded, supercoiled, circular DNA genome of AcMNPV is highly condensed within a nucleocapsid. This condensed packaging is facilitated by the core protein p6.9, a 54 amino acid protein rich in arginine [6]. A major protein of the capsid, vp39, has also been identified. This 39 kDa protein has been found to be distributed randomly over the surface of the nucleocapsid [7].

There are two types of baculovirus progeny, budded virus (BV) and occlusion-derived virus (ODV), which are genetically identical [8]. However, there are differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity [9–13].

BV particles possess spike-like structures known as peplomers, composed of the glycoprotein GP64 for group I NPVs, at one end of the virion [14]. The GP64 protein is incorporated throughout the virus envelope, albeit at lower concentrations than at the peplomers [14]. During infection, GP64 localizes to discrete areas of the plasma membrane at which points budding of virions takes place [15]. Thus, as BV particles bud from the plasma membrane they acquire a plasma membrane-derived envelope containing the GP64 glycoprotein.

Granuloviruses and group II NPVs lack GP64 but instead encode a homolog of another envelope protein, LD130 [16]. While the LD130-encoding baculoviruses do not contain *gp64*

homologs, those encoding GP64 also contain a version of LD130. The OpMNPV LD130 homolog was found to be a glycosylated protein associated with BV particles and the plasma membrane of cells at similar locations as the GP64 protein [17].

One of the major differences between BV and ODV is that the latter do not contain GP64 or LD130. Instead a virus-encoded envelope protein P74 is associated with the outside of the virus [18, 19]. The presence of P74 on the outside of the virion envelope suggests that it may play a role in ODV attachment to midgut cells. Another structural protein of the ODV envelope of *Spodoptera littoralis* (Spli) NPV has been identified. The gene encoding this protein was termed *per os* infectivity factor (*pif*) and is homologous to ORF 119 of AcMNPV [20]. A third gene product inferred to be a structural protein is encoded by *pif-2*, which was identified in *Spodoptera exigua* NPV and is present in AcMNPV as ORF22 [21]. Recent studies have also identified *pif*-4 [22] and *pif*-5 [23, 24] as additional envelope proteins. The PIFs form a complex on the surface of ODV [25].

Other ODV-specific envelope proteins include ODV-E25 [26], ODV-E66 [27], ODV-E56 [28], ODV-E18, and ODV-E35 [29]. These proteins, along with P74, have been found to associate with intranuclear microvesicle structures, which appear in the nucleus during infection [29, 30]. This association has led to the suggestion that these microvesicles play a role in ODV envelopment [27, 28]. Invagination of the inner nuclear membrane observed during baculovirus infection has raised the possibility that it may act as the source of the microvesicles [31–33].

Another ODV-specific protein, GP41, has been identified as an O-linked glycoprotein, predicted to localize to the region between the virus envelope and the nucleocapsid, an area referred to as the tegument [34]. Although GP41 is not present in the budded form of the virus, it has been shown to be required for release of nucleocapsids from the nucleus during BV production [35]. An additional protein, ODV-EC27, has been identified as a structural protein of the ODV envelope and capsid, and may be present in a modified form in BV [29]. This protein may be a cyclin homolog, involved in host cell cycle arrest during baculovirus infection [36]. An envelope protein of both BV and ODV has also been identified (BV/ODV-E26) and found to be associated with intranuclear microvesicles [37]. A seminal study of protein composition of ODV was conducted by using a combination of techniques [38]. Screening expression libraries with antibodies generated to ODV or BV and mass spectroscopic (MS) analysis of ODV protein extracts identified up to 44 potential components unique to ODV. This topic was revisited recently when the protein content of BV was reexamined [39].

The major component of the NPV occlusion body is the 29 kDa polyhedrin protein. While the protein is thought to be small

enough to diffuse through nuclear pores, a nuclear localization signal (KRKK) has been identified at amino acids 32–35 [40]. Another region of the protein (amino acids 19–110) is required for assembly into large occlusion-like particles [40]. The polyhedrin protein is resistant to solubilization except under strongly alkaline conditions and functions to shield virions from physical and biochemical decay while outside the insect host, as well as protecting against proteolytic decay during late stages of infection [41].

Surrounding the polyhedral matrix of a mature occlusion body is the polyhedron envelope (PE), also known as the polyhedron membrane or calyx. The PE was originally reported to be composed of carbohydrate [42]; however, a 34 kDa phosphorylated protein (PP34) has since been found to be covalently bound to the PE of AcMNPV via a thiol linkage [43]. The polyhedron envelope is thought to increase the stability of the occlusion body in the environment and has been found to protect occlusion bodies from physical stress [43, 44]. A recombinant virus unable to produce PP34 was found to be deficient in polyhedron envelope formation and electron dense spacers, which are thought to be precursors of the envelope structure [44, 45].

Both electron dense spacers and the polyhedron envelope have been found in association with fibrillar structures composed of the P10 protein [44, 46]. This protein, like polyhedrin, is produced in large quantities during the occlusion phase. The association of the polyhedron envelope with the P10-containing fibrillar structures suggests a role for the protein in assembly and proper association of the polyhedron envelope around the occlusion body matrix [46]. Comparative analysis of P10 protein sequences revealed that they appear to have an amphipathic alpha-helical terminus that condenses as coiled-coil multimers [47]. The condensation of P10 monomers to coiled-coil multimers may be a step leading to fibrous body formation in virus-infected cells.

## 4 Baculovirus Replication In Vivo

Within a cell, baculoviruses have a biphasic cycle of replication producing two structurally distinct viral phenotypes responsible for specialized functions within the insect host. Occlusion-derived virus (ODV) initiates the primary infection in the gut epithelial cells, while budded virus (BV) is responsible for secondary rounds of infection in the insect host. Infection begins with ingestion of occlusion bodies by a suitable insect host larval stage, followed by dissolution of the occlusion body matrix in the alkaline midgut. Occlusion-derived virus is released from dissolved occlusion bodies, passes through the peritrophic membrane of the midgut and infects the columnar epithelial cells that border the gut lumen. It has been suggested that entry occurs by direct membrane fusion,

involving interaction between virion surface proteins and midgut cell surface proteins [48]. Removal of P74, PIF1 or PIF2 from virus particles by deletion of the appropriate gene has been demonstrated to prevent infection of insect larvae via the oral route [18–21]. Deletion of *pifs*-3-5 has a similar effect [22–24, 49]. Midgut regenerative cells have also been found to be sites of primary infection, albeit at a lower frequency than columnar cells [50]. After replication in the epithelial cells, virus buds in a polar manner, exiting the basal membrane into the hemolymph, thereby allowing the infection to spread throughout the insect host. Early synthesis of the BV envelope fusion protein GP64 seems to allow a "pass through" strategy for the virus, whereby uncoated virus nucleocapsids can rapidly exit the newly infected midgut cells to accelerate the onset of systemic infections [51]. The insect tracheal system has been identified as the main route used by the virus to spread from one tissue to another [52]. Efficient BV formation in AcMNPV requires Ac92 and Ac79 [53, 54].

In addition to enlargement of the nucleus upon baculovirus infection, cells become rounded due to rearrangement of the cytoskeleton. A distinct structure termed the virogenic stroma develops in the nucleus. This is a chromatin-like network of electron-dense filaments. It is the predominant structure in the nucleus from 8 to 48 h postinfection (h pi) [55], and is thought to be the site of viral DNA replication and late gene transcription, as well as nucleocapsid formation [56]. Once assembled, nucleocapsids are released from the nucleus, gaining a nuclear membrane-derived envelope that is subsequently lost during transit through the cytoplasm. In the case of group I NPVs, the envelope glycoprotein GP64 localizes to discrete areas of the plasma membrane during infection and it is at these sites that budding of nucleocapsids takes place [15]. The BV envelope is therefore derived from the plasma membrane and contains the viral glycoprotein GP64. Group II NPVs, and GVs, encode homologs of the envelope fusion (F) protein LD130 [16], which associate with BV particles, and the plasma membrane of infected cells, at similar positions to GP64 in group I NPV-infected cells [17]. These F proteins are functionally analogous to GP64 since they can restore infectivity to mutant viruses lacking *gp64* [57]. GP64 is essential for cell-to-cell BV transmission, since AcMNPV particles containing the protein, but not those lacking the protein, are able to disseminate virus to other cells [58].

Later in infection (~24 h pi), BV production is reduced and nucleocapsids are transported to sites of intranuclear envelopment and incorporation into OBs [44]. The source of the ODV envelope is unclear. A number of ODV envelope proteins have been found to associate with intranuclear microvesicle structures that appear in the nucleus during infection, thereby suggesting they may be the source of the envelope [27–29].

Occlusion bodies accumulate until the terminal stages of infection when the insect liquefies and the occlusion bodies are released into the environment. Liquefaction appears to be caused by the production of both virus-encoded chitinase and cathepsin genes [59–61] and may also involve the *fp25k* product [62]. Deletion of the cathepsin gene from *Bombyx mori* NPV reduced recombinant protein degradation in virus-infected insects, presumably because of the reduction in proteinase activity [63]. Just prior to death many Lepidopteran species crawl to the top of the vegetation on which they were feeding and hang from this elevated position, facilitating dissemination of the virus as the cadaver decomposes [64]. This enhanced locomotory activity (ELA) may well be virus-induced, since insects infected with a *B. mori* NPV mutant lacking the protein tyrosine phosphatase gene showed dramatically reduced ELA before death after about 5 days [65]. However, removal of ecdysteroid glucosyltransferase gene (*egt*) from a baculovirus was found to reduce vertical movement of virus-infected insects on plants [66]. Occlusion bodies serve as survival vehicles for the virus when outside the insect host, as well as acting as dispersal agents between individual insects. They may also protect the virus against proteolytic decay during the end stages of infection [41].

## 5 Baculovirus Gene Expression and Replication

The complete sequence of AcMNPV clone 6 has been determined. The original analysis of the 133,894 bp genome suggested that the virus encodes 154 methionine-initiated, potentially expressed open reading frames (ORFs) of 150 nucleotides or more [67]. However, resequencing of various regions of the genome and comparison with other virus isolates has corrected a number of errors and suggests that AcMNPV may only encode 150 genes. Other baculoviruses have also been sequenced. An up-to-date summary of completed genomes is available from the National Center for Biotechnology Information (NCBI). Baculovirus gene expression is divided into four temporal phases: immediate-early, delayed-early, late, and very late, although the first two are often considered as one. Immediate-early genes are distinguished from delayed-early by their expression in the absence of *de novo* protein synthesis. Expression of delayed-early genes, however, appears to be dependent on the presence of immediate-early gene products. Transcription of late genes occurs concurrently with the onset of viral DNA replication at about 6 h pi. Very late gene transcription begins at about 20 h pi and involves high levels of expression from the polyhedrin and *p10* promoters, two proteins involved in the occlusion of virions. While levels of late gene mRNA transcripts decrease at very late times in infection, very late polyhedrin and p10 gene transcript levels continue to remain high [68].

Analysis of the AcMNPV genome has revealed that RNA transcripts are not clustered according to their temporal expression. Instead, early and late genes are found distributed throughout the genome. All AcMNPV RNAs are both 5′ methyl capped and 3′ polyadenylated [69, 70]. Only one transcript, which is that of the immediate-early gene *ie-1*, is known to be spliced [71]. However, transcriptional units involving overlapping RNAs have been identified in the AcMNPV genome, which may provide an alternative means of introducing a variety of expression [72, 73]. Overlapping transcripts composed of early and late RNAs with a common 3′ end have been identified in a number of regions [72, 73]. The *Hin*dIII-K fragment of the AcMNPV genome produces five overlapping RNAs, two immediate-early, one delayed-early, and two late gene transcripts, transcribed in the same direction and terminating at a common 3′ end [73]. This arrangement has been implicated in the temporal regulation of these genes. It has been suggested that the longer, later 5′ extended transcripts serve to repress transcription of earlier genes, located downstream, probably by means of promoter occlusion, as well as acting as mRNAs for late viral products [73].

The polyhedrin and p10 genes are also transcribed as several overlapping RNAs. In the case of these two genes, however, transcripts have common 5' ends, with longer RNAs being derived from read through of termination signals at the 3′ end of the smaller RNAs [72–74]. Four overlapping transcripts were mapped to the p10 gene region and were found to comprise two sets: a late phase pair of transcripts (1100 and 1500 bases) sharing a common 5′ end that are most abundant at 12 h pi and a very late phase pair (750 and 2500 bases) that also have a common 5′ end and are most abundant at 24 h pi [74]. Promoter occlusion may also be operating in this region. Synthesis of earlier transcripts may prevent RNA polymerase initiation at the *p10* promoter located downstream. At later times, when transcription from upstream promoters has ceased, RNA polymerase may be able to initiate at the downstream *p10* promoter [74].

### 5.1 Early Gene Expression

Baculovirus early genes are transcribed before the onset of viral DNA replication. Expression of many early genes begins immediately after cell infection, with some transcripts, for example the anti-apoptotic p35 gene early mRNAs, appearing within the first 2 h [75]. Genes in this phase of the virus lifecycle are transcribed by the host RNA polymerase II, as demonstrated by the fact that early transcription is sensitive to α-amanitin [76], a compound that binds to and inactivates the large subunit of RNA polymerase II.

#### 5.1.1 Promoter Elements

Most early baculovirus promoters contain a TATA element which, as well as regulating the rate of transcription initiation, also establishes

the position of the RNA start site 25–30 bases downstream [71, 77–79]. An initiator motif (ATCA(G/T)T(C/T)), which overlaps the RNA start site, has also been identified in many early promoters. The most conserved of these is the CAGT motif that has been found to contribute to basal promoter activity, as well as being sufficient in determining the position of the RNA start site in the absence of a TATA element [79–82]. In composite promoters, those containing both CAGT and TATA motifs, cooperation is thought to occur between the two to stabilize host transcription machinery or enhance recruitment of required factors [83].

The early promoter of *dnapol*, which encodes the viral DNA polymerase, does not contain a TATA or CAGT motif. Early transcription from this promoter initiates from multiple sites including the sequence CGTGC [84]. This sequence is also found at the start of *p143* [85]. The significance of this unusual promoter motif is unknown, although it is thought that these promoters may be more responsive to viral transactivators during infection [83].

Another *cis*-acting element identified in early promoters is a downstream activating region (DAR). A DAR has been identified in the 5′ noncoding regions of the immediate-early *ie-1* gene [84]. This element, located between positions +11 and +24, is necessary for optimal expression of *ie-1* early in infection [81]. The core DAR sequence (A/T)CACNG has also been identified in the 5′ noncoding region of the envelope glycoprotein encoding gene *gp64*, and has been found to stimulate the rate of early *gp64* transcription [81, 86].

Many early baculovirus promoters have also been found to possess an upstream activating region (UAR), consisting of one or more *cis*-acting DNA elements that affect the level of transcription, but not the position of the RNA start site [78, 79, 87]. The *ie-1* UAR has been found to increase promoter activity twofold early in Sf-21 cell infection [81], while the *p35* UAR was shown to be responsible for a 10- to 15-fold enhancement of basal transcription [78]. A number of distinct UAR elements have been identified. The first consists of GC-rich sequences and, as such, is termed the GC motif. GC motifs have been found in the UARs of a number of early baculovirus promoters including those of *p35* and *39K*, a gene encoding a protein (pp31) associated with the virogenic stroma [78, 79, 88]. The CGT motif consists of the consensus sequence A(A/T)CGT(G/T) and has been identified in the UARs of *p35*, *39K* and the helicase encoding gene *p143* [78, 79, 85]. A third UAR motif, referred to as the GATA element, has also been identified in the early *gp64* promoter and in that of the immediate-early *pe-38* [86, 89].

#### 5.1.2 Transcriptional Enhancers

The AcMNPV genome contains homologous regions (*hrs*), rich in *Eco*RI sites, distributed throughout the genome [90]. Eight of these regions (*hr*1, *hr*1a, *hr*2, *hr*3, *hr*4a, *hr*4b, *hr*4c, *hr*5) have been

identified and were found to consist of two to eight copies of a 28 bp imperfect palindromic repeat (28-mer) bisected by an *Eco*RI site and flanked on each side by direct repeats of about 20 bp [67, 91]. Several early viral promoters, including those of *39K*, the immediate-early gene *ie-2* (formerly *ie-n*), *p143* and *p35*, have been shown to be stimulated by *cis*-linkage to *hrs* [80, 85, 87, 92–94]. Promoter enhancement by *hrs* occurs in a position- and orientation-independent manner [93] and this enhancement is further augmented by the immediate-early IE-1 protein [91], a viral transactivator which has been found to bind to *hr* sequences [94–96]. The *hr* 28-mer is the minimal sequence required for IE-1 mediated promoter enhancement [92–94]. The IE-1 protein binds to the 28-mer as a dimer, interacting with the two palindromic half-sites, both of which are required for *hr* enhancer activity [93–96]. Oligomerization of IE-1 is thought to occur in the cytoplasm, before localization to the nucleus, binding to *hr* sequences, and subsequent enhancement of promoter activity through interaction with components of the basal transcription complex [95, 97].

#### 5.1.3 Transactivational Regulators

A number of transactivational regulators of baculovirus early gene promoters have been identified. The immediate-early gene, *ie-1,* is thought to be the principal transregulator of early baculovirus expression and was originally identified due to its *trans*-acting regulatory role in *39K* expression [92]. As well as stimulating expression of genes such as *p35*, *p143*, and *39K* [85, 87, 92, 98], IE-1 is capable of stimulating its own promoter [99]. The N-terminal region of the 582 residue IE-1 protein has been found to contain a transactivation domain, while the C-terminal of the protein contains a DNA binding domain [95]. A small basic domain between residues 537 and 538 has been identified as a nuclear localization signal, which functions upon dimerization of IE-1 [97]. Transcripts of *ie-1* give rise to both spliced and unspliced RNAs. Unspliced transcripts encode IE-1 itself, while spliced transcripts encode another immediate-early transregulator, IE-0, identical to IE-1 except for 54 additional amino acids at its N-terminus [71, 99]. While IE-0 is expressed only during the early phase of infection, IE-1 RNA is expressed in both the early and late phases [71]. Transient expression assays have shown IE-1 to have a negative regulatory effect on *ie-0* promoter expression, while IE-0 transactivates the *ie-1* promoter [99]. Deletion of *ie-1/ie-0* from the virus genome using an *Escherichia coli*-based system prevented virus replication in insect cells, although restoration of the mutant with either gene largely restored production of infectious virus progeny [100].

The transcriptional regulator IE-2 indirectly stimulates expression from promoters dependent on IE-1 for transactivation, by increasing transcription from the *ie-1* promoter. The protein has been shown to be capable of enhancing IE-1 transactivation of both

the *p143* and *39K* promoters [85, 101]. In addition to enhancing IE-1 expression, IE-2 stimulates expression of IE-0 in transient assays, as well as auto-regulating its own expression [80]. Like IE-0 expression, IE-2 regulation has been shown to be down-regulated by IE-1 [102]. Another transactivational regulator, encoded by *orf121*, has been shown to stimulate the *ie-1* promoter in a similar manner to IE-2 in transient assays [103].

The immediate-early gene *pe-38* encodes a 38 kDa protein, which also acts as an early transregulator. The p143 gene promoter is transactivated by PE-38, and this transactivation has been found to be augmented by IE-2 [85]. However, the delayed-early *39K* promoter was not stimulated by PE-38 [85], suggesting that PE-38 has a restricted transactivation range compared to IE-1, which is capable of stimulating both promoters [85, 92].

Transcripts of *ie-0*, *ie-2* and *pe-38* are expressed during the early phase of infection, while *ie-1* RNAs are expressed during the early and late phases. The fact that IE-1 down-regulates expression of both IE-0 and IE-2 in transient assays suggests that it may function to shut off immediate-early gene expression during the late phase of infection [99, 102], while IE-1 stimulation of its own promoter suggests that it positively regulates its own expression during infection [99]. Baculovirus *pe-38* is transactivated by IE-1 when both are transfected into mammalian BHK-21 cells [104].

### 5.2 Baculovirus and Apoptosis

As one of the first viral families found to be capable of regulating host apoptotic pathways, the baculoviruses have become important tools in the study of apoptosis [105, 106]. Apoptosis may have evolved to remove unwanted cells in the development of an organism but has been adapted as an antiviral defense mechanism. Members of the *Baculoviridae* encode a number of important apoptotic suppressors. The study of the mechanism of action of these proteins in baculovirus-infected insect cells has revealed important information about conserved points in the cell death pathway. Additional information about the use of baculoviruses in the study of apoptosis can be found in Chapter 25 of this book.

AcMNPV infection of Sf-21 cells induces apoptosis, thereby resulting in the activation of the novel insect caspase SF-caspase-1, an effector caspase found to have sequence similarity to human caspase-3,-6, and-7 [107]. Expression of the AcMNPV anti-apoptotic p35 gene blocks apoptosis allowing replication of the virus to proceed [105]. The specific factors that activate the cell death pathway in AcMNPV-infected Sf-21 cells are unclear. While budded virus binding alone is not sufficient to induce apoptosis, transient expression of the IE-1 protein has been found to induce cell death in Sf-21 cells [108]. Cell death induced by IE-1 is further enhanced by *pe-38* in transient assays, although the precise mechanism by which this augmentation takes place remains unclear [109].

Although some induction of apoptosis occurs upon IE-1 expression, viral DNA replication is required for the full apoptotic response [110, 111]. This is supported by the finding that the timing of DNA synthesis coincides with activation of SF-caspase-1 and the occurrence of the first morphological signs of apoptosis, such as cell membrane blebbing [110]. It is possible that DNA synthesis induces apoptosis indirectly by promoting the onset of late gene expression. Alternatively, viral DNA replication may activate apoptosis directly by damaging cellular DNA or disturbing the insect cell cycle.

The baculovirus anti-apoptotic p35 gene was first identified during characterization of an AcMNPV spontaneous mutant. The mutant, termed the annihilator (vAcAnh), was found to cause premature death in *S. frugiperda* (Sf-21) cells, but not in *T. ni* (Tn-368) cells [105]. Infection of Sf-21 cells with wild type AcMNPV causes transient plasma membrane blebbing at approximately 12 h pi [105]. A similar effect was observed with vAcAnh infection of Sf-21 cells. However, while this blebbing disappeared in the wild type infection, it was found to intensify with the mutant infection, thereby resulting in disintegration of cells into apoptotic bodies [105]. Cell blebbing was not observed in *T. ni* cells infected with either the wild type virus or vAcAnh, allowing amplification of the mutant in this cell line [105]. Annihilator mutant-infected Sf-21 cells also exhibited a number of other features of apoptosis, including nuclear condensation, intact mitochondria retention until late in the apoptotic process, and internucleosomal cleavage of cellular DNA beginning between 6 and 12 h pi [105]. A deletion in the p35 gene, located in the *Eco*RI-S fragment of the AcMNPV genome, was subsequently identified as being responsible for the annihilator mutant phenotype [105]. The p35 gene is transcribed from a promoter containing both early and late start sites, although it is predominantly transcribed as an early gene, with transcripts detectable within the first 2 h of infection [75, 78, 87]. The gene encodes a 299 amino acid protein with no recognizable sequence motifs.

Another class of anti-apoptotic genes, the inhibitor of apoptosis (*iap*) genes, has also been identified. Baculovirus IAP proteins block apoptosis in Sf-21 cells induced by a number of different stimuli other than baculoviral infection, including treatment with apoptosis inducing agents such as actinomycin D, cycloheximide, tumor necrosis factor α and UV light [112]. A *Cydia pomonella* granulovirus (CpGV) iap gene product, Cp-IAP-3, was the first IAP protein to be identified and confirmed to have anti-apoptotic activity. This Cp-iap-3 gene was initially identified during a genetic screen for genes that could complement the absence of p35 in annihilator mutant-infected Sf-21 cells. Cp-IAP-3 is expressed both early and late in infection from distinct transcription start sites and has been found to localize in the cytoplasm, with no

IAP-3 detectable in the nuclei of infected cells [113]. Five iap genes have been recognized: iap-1 is found in Group I NPVs, iap-2 occurs in both Group I and II NPVs, iap-3 is encoded by Group I, II, GVs and hymenopteran NPVs, whereas iap-4 is present in a few Group I and II NPVs and iap-5 occurring solely in GVs [114].

A characteristic of all members of the IAP family is the presence of 1–3 copies of an imperfect 70 amino acid repeat called a Baculovirus IAP Repeat (BIR) at the N-terminus of the protein [106]. Baculoviral IAPs, and several cellular IAPs, also contain a carboxy terminal RING finger (zinc-like-finger) motif. Both BIR and RING domains are thought to be involved in protein–protein interactions and are essential for inhibition of apoptosis, with the BIRs having been implicated directly in the binding and inhibition of caspases [115]. The mechanism by which baculovirus IAPs block apoptosis is distinct from that of P35. While P35 is capable of directly interacting with and inhibiting the active caspase, IAP acts upstream of this by inhibiting maturation of the procaspase [116].

### 5.3 Baculovirus Replication

The homologous regions (*hrs*), identified as enhancers of early gene expression, have also been proposed as origins of viral DNA replication [90]. Evidence for this role was obtained through assays of transient replication, which have shown plasmids containing *hrs* to be capable of AcMNPV dependent replication when these were used to transfect Sf-21 cells [117]. As with early promoter enhancement, a single 28-mer is sufficient to support plasmid replication [94]. However, deletion mutagenesis of *hr5*, which contains six palindromes, revealed the efficiency of replication from individual *hrs* to be dependent on the number of palindromes presents [118]. An AcMNPV non-*hr* containing origin has also been identified in the *Hin*dIII-K fragment of the genome [117]. A circular topology has been found to be a requirement for replication of origin-containing plasmids, suggesting the mechanism of baculovirus DNA replication involves a theta or rolling circle intermediate [117].

Six genes, encoding P143 (DNA helicase), DNA polymerase [119], IE-1, late expression factor-1 (LEF-1) (primase), LEF-2 (primase associated protein) [120], and LEF-3, (single-stranded DNA binding protein) [121, 122] have been found to be essential for transient DNA replication [123]. In addition, *lef-11* was reported to be essential for AcMNPV replication in Sf-9 cells [124]. DNA-independent ATPase activity has been associated with the DNA helicase of *Trichoplusia ni* granulovirus [125]. Deletion of the DNA polymerase gene from the virus genome abrogates virus replication [126]. LEF-2 is a capsid protein not required for initiation of DNA replication but is necessary for amplification [127].

Genes encoding P35, IE-2, PE-38 and LEF-7 are thought to play a stimulatory role in DNA replication [123, 128]. The stimulatory effect of the anti-apoptotic *p35* gene in transient assays is

thought to be largely due to its role in preventing premature cell death, although there is evidence suggesting that P35 may also be involved in early gene regulation [98]. The *pe-38* gene product has been seen to play a role in the activation of expression of the baculovirus helicase homolog P143, while IE-2 stimulates *pe-38* and *ie-1* expression [85, 129]. Stimulation of viral DNA replication by LEF-7 was observed [128] and this protein has been found to contain two single stranded binding protein (SSB) motifs.

Baculovirus DNA replication is associated with distinct foci in the nuclei of infected cells [130]. It was found that IE-2, LEF-3 and an additional protein thought to play a role in AcMNPV replication, termed DNA binding protein (DBP), colocalized with centers of viral DNA replication within the nucleus [130]. The importance of LEF-3 in DNA replication is further underlined by its involvement with viral helicase and polymerase. The protein interacts with P143 and is required for nuclear localization of the helicase [131, 132]. It has also been found to play a role in enhancing the strand displacement activity of DNA polymerase [119]. Interaction between LEF-1 and LEF-2 has been observed, and is thought to be required for DNA replication [133, 134]. The IE-1, LEF-3 and P143 products interact with DNA in vivo, as demonstrated by formaldehyde cross linking studies [135]. Deletion of very late factor 1 (vlf1) reduces DNA replication to a third of normal levels and no budded virus is produced [136]. Virus DNA levels double every 1.7 h from 6 h pi until about 20 h pi [137]. By this point each cell contains about 84,000 genomes.

## 5.4 Late and Very Late Gene Expression

### 5.4.1 Viral RNA Polymerase

The RNA polymerase responsible for transcription of late and very late baculovirus genes is encoded by the virus itself [138]. This viral polymerase is α-amanitin-resistant and unable to transcribe from early gene promoters [139]. The products of four lef genes, *lef-8*, *lef-4*, *lef-9* and *p47*, have been identified as components of the AcMNPV RNA polymerase [138]. The LEF-4 has guanylytransferase activity [140] and RNA 5′ triphosphatase and nucleoside triphosphatase activity [141]. Genes encoding each of the RNA polymerase subunits have been shown to be necessary for late and very late gene expression in transient expression assays [128, 142, 143]. Studies on temperature sensitive mutants had previously identified similar roles for p47 and LEF-4 in late gene transcription, suggesting them to be members of the same protein complex [144]. Pairwise interactions between LEF-9 and P47, LEF-4 and P47, and LEF-8 and P47 have been demonstrated, but interactions between LEF-4 and LEF-8 do not occur unless P47 is present [145].

The viral RNA polymerase is thought to carry out both 5′ methyl capping and 3′ polyadenylation of late and very late transcripts [140, 141, 146]. The RNA triphosphate cleaves the 5′-triphosphate from primary transcript termini, producing the guanylyltransferase substrate. The final stage of the reaction

requires RNA methyltransferase to catalyze the transfer of a methyl group to the guanosine cap. The protein responsible for this stage of the process remains to be confirmed, although it has been suggested that a host cell enzyme could carry out this step [138, 141]. LEF-4 has also been found to possess an ATPase activity, although the function of this activity is unknown [146]. Both LEF-8 and LEF-9 contain conserved motifs present in the large subunits of other DNA-directed RNA polymerases [146, 147]. The conserved motif of LEF-8 had been proposed as a putative catalytic site of the enzyme [146]; however, *lef-8* mutagenesis studies have revealed that both termini of the protein are essential for its function [148]. In addition, a host cell protein, designated polyhedrin promoter binding protein (PPBP), has been identified that binds to very late promoters and is required for expression of these genes [149, 150]. The protein has both double stranded and single stranded binding activities and may act as an initiator binding protein similar to the TATA-binding protein (TBP) required for transcription initiation by eukaryotic RNA polymerases I, II and III [150].

#### 5.4.2 Promoter Elements

Baculovirus late and very late transcripts initiate at the central A of a conserved (A/G)TAAG sequence, usually ATAAG for abundant transcripts, which is essential for promoter activity [6, 7, 151]. Analysis of the *gp64* promoter region, which contains two active and three inactive TAAG motifs, revealed that sequences immediately surrounding the initiation site, rather than its position, determine its use as a late gene promoter [152]. Mutations in sequences surrounding TAAG have been shown to reduce transcription at the level of transcription initiation [153]. However, the TAAG motif and its surrounding sequences are not involved in mediating the difference in temporal regulation between late and very late transcription. The very late polyhedrin and p10 genes are abundantly expressed at very late times in the infection. Despite having little homology, the 5′ leader sequences of both promoters are extremely A+T rich and contain sequences necessary for the burst in expression observed very late in infection [153–156]. Mutations upstream of the TAAG motifs of both very late promoters exert a mild effect on expression [154, 157]. Thus, the polyhedrin promoter consists of a 49 bp 5′ untranslated region and 20 bp upstream of the transcription start site [154], while the *p10* promoter consists of a 70 bp 5′ untranslated region and 30 bp upstream of the transcription start site [155, 157]. Polyhedrin and *p10* promoters appear to be regulated differently, with *p10* expression occurring earlier in infection and at lower levels than that of polyhedrin [158].

#### 5.4.3 Regulation of Late and Very Late Gene Expression

Nineteen Late Expression Factor (*lef*) genes of AcMNPV, which are necessary and sufficient for transient expression from both late and very late viral promoters in Sf-21 cells, have been identified [143, 159–161]. A subset of these genes is involved in plasmid

DNA replication, while another four are thought to be subunits of the viral RNA polymerase, as outlined above. The remaining *lefs* (*lef-5, lef-6, lef-10, 39K, lef-12*) are thought to function in late promoter recognition or stabilization of late transcripts [128]. In addition to its role in plasmid DNA replication, *ie-1* is thought to have a direct effect on expression from the very late polyhedrin promoter [162]. Further genes involved in transient late gene expression have been identified [161]. One of these genes, termed *orf41*, was found to be necessary for late gene expression, while *orf69* was found to play a stimulatory role [161]. LEF-12 is dispensable for virus replication [163]. It was suggested that IE0 could be regarded as the 20th LEF since it can replace IE1 in a transient replication assay [164].

The FP25K protein, a late gene product thought to be a structural component of the nucleocapsid, enhances the rate of transcription from the very late polyhedrin promoter, but not that of the *p10* promoter [165]. The effect of this protein on transcription of only one of these very late hyperexpressed genes is consistent with the differential regulation observed for the two promoters [158].

The Very Late Expression Factor-1 (*vlf-1*), is specific for regulation of very late transcripts [166]. The encoded protein, VLF-1, is required for high level expression from the polyhedrin and *p10* promoters and is thought to exert its effect by interacting with the burst sequences located between the transcriptional and translational start sites of each promoter [167]. Although *vlf-1* is mainly transcribed at late times in infection, the stability of the protein allows it to remain at high levels throughout the very late phase [168, 169]. Accelerated production of VLF-1 results in premature polyhedrin synthesis, showing that the timing of VLF-1 expression is important in very late gene transcription [169].

VLF-1 has also been found to play a role in BV production, possibly as a resolvase or topoisomerase to produce monomeric viral genomes from concatemeric products of the DNA replication process [168]. The predicted sequence of the 44.4 kDa VLF-1 protein shows similarity to the sequences of a large class of resolvases and integrases found in *Saccharomyces cerevisiae*, prokaryotes and phages [166]. Viruses carrying *vlf-1* null mutations have been found to be either nonviable or so defective in BV production that they were extremely difficult to propagate, thereby indicating an essential role for the protein in baculovirus replication [168]. The level of VLF-1 protein required for activation of very late promoter burst sequences is thought to be much higher than is required for the protein's role in BV production [169]. Overexpressed and purified VLF1 added to transcription assays containing baculovirus RNA polymerase stimulated transcription of the polyhedrin gene promoter, but not 39K [170].

Serial passage of Nucleopolyhedroviruses through cultured cell lines results in the appearance of a spontaneous mutant termed the 'few polyhedra' (FP) mutant [171, 172]. With continued passage this FP phenotype becomes dominant [173]. Fourteen passages of *Trichoplusia ni* (Tn)MNPV in *T. ni* cells was found to result in a purely FP mutant population [173]. The characteristics most commonly associated with the FP phenotype are a reduced number of polyhedra per cell compared to the wild type, occlusions containing no virions or virions of altered morphology, altered intranuclear envelopment, and the production of more BV than cells infected with the wild type [172–174].

A common feature of many AcMNPV and *Galleria mellonella* (Gm)MNPV FP mutants is insertion of DNA sequences (0.8–2.8 kb), homologous to moderately repetitive host DNA, into a region of the genome encoding a 25 kDa protein (i.e., the FP25K protein). Subsequent studies correlated AcMNPV FP mutations to large insertions of host cell DNA or deletions of viral DNA, detectable by restriction endonuclease (RE) analysis, in this region of the genome [175, 176]. Targeted mutation of AcMNPV *fp25k* confirmed alterations in this gene to be sufficient to cause the complex characteristics of the FP phenotype, including reduced virion occlusion, altered intranuclear envelopment and enhanced BV production [177].

A late gene product, the FP25K protein, is highly conserved among members of the Nucleopolyhedroviruses, with the last 19–26 C-terminal amino acids the only region lacking significant conservation among sequenced *fp25k* genes [178]. Although identified as a structural protein of the nucleocapsids of BV and ODV [179], a large fraction of the protein remains associated with amorphous cytoplasmic bodies throughout infection [179].

Mutations in *fp25k* alter the apparent expression and/or accumulation of several viral proteins. Rates of both polyhedrin biosynthesis and nuclear localization are reduced in cells infected with *fp25k* mutants [180]. The effect of FP25K on polyhedrin biosynthesis was found to occur at the level of transcription, with wild type FP25K found to enhance expression from the polyhedrin promoter [165]. While the rate of polyhedrin transcription in *fp25k* mutants was reduced, *p10* RNA levels were unaffected, suggesting that the reduction in polyhedrin RNA does not reflect a general effect on very late gene expression [165]. Acquisition of mutations within *fp25k* in baculovirus expression vectors could, therefore, seriously affect recombinant protein production. This might occur if recombinant virus is produced and amplified in *T. ni* cell lines. Thus, most commercial baculovirus expression systems guard against using *T. ni* cells for virus production and amplification, recommending these cells for protein production only.

## 6 Baculoviruses as Expression Vectors

Two features of baculoviruses underpin their use as expression vectors. The first is that the very late polyhedrin and p10 genes are dispensable for virus replication in cell culture and in insects if the budded virus is delivered to the hemocoel of the larval host [180–182]. The second is that both of these virus gene promoters are very strong and if coupled with a foreign gene coding region can enable the production of large amounts of recombinant protein in insect cells. Recent studies to elucidate the nature of the very late virus gene promoters have also enabled expression vectors to be derived that contain multiple copies of the polyhedrin and p10 promoters so that several recombinant proteins can be produced simultaneously in virus-infected cells [183–185]. These vectors have been of particular use in assembling structures in insect cells that are composed of more than one protein [186]. Insect cells are also competent in accomplishing many of the post translational processes required when producing proteins from eukaryotic cells, thereby producing biologically active products.

The original method for producing recombinant baculoviruses required replacing the native polyhedrin gene with the heterologous coding sequences, thus deriving a polyhedrin-negative virus. This virus had to be identified by visual selection of plaques lacking polyhedra in a standard virus titration. While moderately difficult to the experienced user, it often proved impossible for the novice. Fortunately, this problem has been solved by a wide variety of newer methods that enable modification of the virus genome to be done more easily. Currently, automated systems for making recombinant baculoviruses are being devised that promise to facilitate the simultaneous production of dozens, if not hundreds, of expression vectors. Baculoviruses have also become a useful tool for introducing foreign genes into human cells, where the lack of virus amplification means that there need be no concerns over biosafety of the gene delivery vector [187].

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# Chapter 3

# Baculovirus Transfer Vectors

**Robert D. Possee and Linda A. King**

## Abstract

The production of a recombinant baculovirus expression vector normally involves mixing infectious virus DNA with a plasmid-based transfer vector and then co-transfecting insect cells to initiate virus infection. The aim of this chapter is to provide an update on the range of baculovirus transfer vectors currently available. Some of the original transfer vectors developed are now difficult to obtain but generally have been replaced by superior reagents. We focus on those that are available commercially and should be easy to locate. These vectors permit the insertion of single or multiple genes for expression, or the production of proteins with specific peptide tags that aid subsequent protein purification. Others have signal peptide coding regions permitting protein secretion or plasma membrane localization. A table listing the transfer vectors also includes information on the parental virus that should be used with each one. Methods are described for the direct insertion of a recombinant gene into the virus genome without the requirement for a transfer vector. The information provided should enable new users of the system to choose those reagents most suitable for their purposes.

**Key words** Baculovirus, Polyhedrin gene, p10 gene, Single/multiple expression vectors, Fusion vectors, Biotechnology, Molecular biology, Virology

## 1 Introduction

The range of available baculovirus transfer vectors is vast, with many variants of a basic design. Since the introduction of the baculovirus system [1, 2], transfer vectors for single gene, multiple gene, and fusion gene expression have been developed by many laboratories. These sources include both academic and commercial groups. In contrast to the early days of baculovirus expression systems, many transfer vectors and complete kits are available commercially. The days of requesting particular vectors from an academic laboratory are largely gone, although many interesting plasmids can be unearthed if one has the patience to sift through the available literature. Instead the preferred route is often to purchase what is required from one of the companies offering baculovirus expression products. Frequently, these bodies have developed vectors with features, such as multiple cloning sites or

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convenient systems for exchange of genes between plasmids, which make the system easier to use. Therefore, this chapter refers to reagents that are only available commercially. Inevitably, some companies supply broadly similar plasmids. We have avoided placing a recommendation on any one source, leaving it to the reader to make their choices based on the subtleties of different vector design. Given the multitude of plasmids and selection systems available, it is not possible to list every transfer vector that is potentially of use to the reader. It is also not feasible to present a timeline of transfer vector developments. Instead, we provide guidance on transfer vectors that can be used for all of the major applications of the baculovirus system. This chapter focuses on the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), although we acknowledge that *Bombyx mori* NPV has much to offer as an expression vector system, particularly for in vivo production of recombinant proteins in silkworm larvae [3]. Experimental protocols are not included in this chapter. The emphasis is on transfer vector selection with the appropriate parental virus.

### 1.1 The Role of the Baculovirus Transfer Vector

The sole purpose of the baculovirus transfer vector is to enable the insertion of foreign gene coding regions, under the regulatory control of an appropriate gene promoter, into the virus genome at a site that will not affect normal virus replication. It was necessary to devise this strategy as the baculovirus genome is large (ca. 134 kilobase pairs [134 kbp]) [4] and direct ligation with foreign DNA, although feasible [5, 6], is difficult. Other strategies have involved enzymatic recombination in vitro [7] or homologous recombination and selection in yeast [8]. Although these methods are ingenious, neither has become part of the mainstream baculovirus technology.

The baculovirus transfer vector comprises a portion of the virus genome that spans the intended site for insertion of the foreign gene. Most commonly, this region contains the polyhedrin [1] or p10 [9] genes. Both are dispensable for virus replication in cell culture or insect larvae. Both genes, however, are very highly expressed in the very late phase of virus replication [10]. Their promoters are well characterized and effect high level transcription of recombinant sequences after insertion back into the virus genome. A typical baculovirus transfer vector comprises a polyhedrin or p10 gene promoter, a transcription terminator, most often that of the native virus gene and regions on both sides of the promoter that are homologous to the target in the virus genome. The sequences between the promoter and transcription terminator may comprise multiple restriction enzyme sites to facilitate insertion of the foreign coding region. Additional sequences may include signal peptide or other peptide tag coding regions to facilitate protein secretion and purification, respectively. The great advantage of using a transfer vector is that it is easily manipulated in vitro and the correct insertion of a foreign gene can be verified using techniques with which most scientists are familiar. Advances in DNA

sequencing mean that it is easy to determine the primary genetic structure of the recombinant molecule.

Subsequent to the construction of a transfer vector, it is mixed with virus DNA and used to co-transfect insect cells to establish a virus infection. Within the virus-infected cells, recombination occurs between the homologous sequences in the plasmid transfer vector and the virus genome. The native virus gene, most often polyhedrin, is removed in a double crossover event and replaced with the foreign coding region to be expressed in the insect cell. Subsequent selection of the recombinant virus may be accomplished using a variety of techniques (*see* below).

## 1.2 The Parental Baculovirus Genome

Any description of baculovirus transfer vectors must also include an account of the recipient parental virus genome into which the foreign gene is to be inserted. Some sets of vectors are only compatible with certain parental virus DNAs, so care must be taken when deciding on a particular approach. If supplies of viruses and vectors are obtained or purchased from a single source, then few difficulties should arise. However, attempting to mix and match different reagents may cause problems. Some different baculovirus selection systems are available from the same company, so there is the potential for confusing transfer vectors and parental virus genomes. The list of commonly used transfer vectors provided in Table 1 are grouped according to the type of parental DNA that must be used to make a recombinant baculovirus, rather than by individual features of the plasmids, such as promoters or cloning sites. The rationale for this presentation is that users have to make an initial decision when first employing baculovirus technology as to the system that is most appropriate for their needs. The widest range of transfer vectors is still those available for use with infectious virus DNA to make recombinant viruses after co-transfection of insect cells.

### 1.2.1 Circular Virus DNA

The earliest studies with baculovirus expression vectors utilized circular virus genomes as the recipient of the desired foreign gene sequences. The principle of the method was the replacement of the native polyhedrin gene with the foreign DNA, so that the recombinant virus produced no longer synthesized polyhedrin protein and in consequence was unable to assemble occlusion bodies or polyhedra in virus-infected cells [1, 2, 11]. The major problem with this approach was that the proportion of the progeny virus population derived from the co-transfection experiment could be less than 0.1 % [11]. This required a very difficult search for polyhedra-negative plaques after staining good quality titrations in insect cells. A good quality microscope and an experienced eye were also essential. Screening for more than five recombinant viruses simultaneously was almost impossible because of the number of titrations required. This system was not amenable to non-specialist use of baculovirus expression vectors.

**Table 1**
**Baculovirus transfer vectors and parental viruses**

| Type (*locus*) | Vector (size) | Promoter(s) | Features within cloning sites and poly(A) signal (PA) | Additional features/ notes | Source | Parental virus (Source) |
|---|---|---|---|---|---|---|
| Single promoter (*polyhedrin*) | pFastBac™1 (4.8 kbp) | *Polyhedrin* | *Bam*H I, *Rsr* II, *Bss*H II, *Eco*R I, *Stu* I, *Sal* I, *Sst* I, *Spe* I, *Not* I, *Nsp*V, *Xba* I, *Pst* I, *Xho* I, *Sph* I, *Kpn* I, *Hin*d III | | Life Technologies | Bac-to-Bac maintained in *E. coli*. (Life Technologies) |
| | pFastBac™/ HBN-TOPO (4.8 kbp) | | ATG, Honeybee melittin secretion signal, TOPO insertion site, TEV protease cleavage site, 6× His | | | |
| | pFastBac™HT A, B, C (4.8 kbp) | | ATG, 6× His tag-TEV protease cleavage site, *Ehe* 1, *Nco* I, *Bam*H I, *Eco*R I, *Stu* I, *Sal* I, *Sst* I, *Spe* I, *Not* I, *Nsp* V, *Xba* I, *Pst* I, *Xho* I, *Sph* I, *Kpn* I, *Hin*d III | Frameshift occurs at the *Bam*H I site in each vector | | |
| Multiple promoter (*polyhedrin*) | pFastBac™Dual (5.2 kbp) | *Polyhedrin*<br>*p10* | *Bam*H I, *Rsr* II, *Bss*H II, *Eco*R I, *Stu* I, *Sal* I, *Sst* I, *Spe* I, *Not* I, *Nsp* V, *Xba* I, *Pst* I, *Hin*d III<br>*Bbs* I, *Sma* I, *Xho* I, *Nco* I, *Nhe* I, *Pvu* II, *Nsi* I, *Sph* I, *Kpn* I | Dual expression from polyhedrin and p10 gene promoters | | |
| Single promoter (*polyhedrin*) | pDEST™8 (6.5 kbp) | *Polyhedrin* | Gateway™ entry site | Insert genes from Gateway™ entry vectors, such as pENTR™ | | |
| | pDEST™10 (6.7 kbp) | | Gateway™ entry site<br>N-term. 6× His tag, TEV<br>Gateway™ entry site | | | |
| | pDEST™20 (7.0 kbp) | | N-term. Glutathione-S-transferase (GST) | | | |

| | | | | |
|---|---|---|---|---|
| pVL1392 (9.6 kbp) | *Polyhedrin* | *Bgl* II, *Pst* I, *Not* I, *Eag* I, *Eco*R I, *Xba* I, *Sma* I, *Bam*H I | BD Biosciences | AcMNPV (wild type virus DNA)<br>BacPAK6 (13; Clontech)<br>BaculoGold (BD Biosciences)<br>Bac1000<br>Bac2000<br>Bac3000<br>BacMagic™<br>BacMagic™-2<br>BacMagic™-3<br>(EMD Millipore)<br>*flashBac*™<br>Cloned version of AcMNPV unable to replicate in insect cells unless rescued with appropriate transfer vector.<br>(Oxford Expression Technologies)<br>Sapphire™ DNA contains protein disulphide isomerase gene at p10 locus for enhanced protein folding.<br>(Allele)<br>ProGreen<br>ProFold<br>ProEasy<br>(AbVector) |
| pVL1393 (9.6 kbp) | *Polyhedrin* | *Bam*H I, *Sma* I, *Xba* I, *Eco*R I, *Not* I, *Eag* I, *Pst* I, *Bgl* II | | |
| pAcG2 (8.5 kbp) | *Polyhedrin* | ATG, GST, Thrombin cleavage-*Bam*H I, *Sma* I, *Eco*R I | | |
| pAcG3X (8.5 kbp) | *Polyhedrin* | ATG, GST, Factor Xa cleavage, *Bam*H I, *Sma* I, *Eco*R I | | |
| pAcGHLT-A, (8.7 kbp) | *Polyhedrin* | ATG, GST, *Bam*H I-6× His tag, Protein kinase A site, Thrombin cleavage-*Nde* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | | |

(continued)

**Table 1**
**(continued)**

| Type (*locus*) | Vector (size) | Promoter(s) | Features within cloning sites and poly(A) signal (PA) | Additional features/ notes | Source | Parental virus (Source) |
|---|---|---|---|---|---|---|
| | pAcGHLT-B, (8.7 kbp) | *Polyhedrin* | ATG, GST, *Bam*H I, 6× His tag-Protein kinase A site, Thrombin cleavage-*Xho* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | | | |
| | pAcGHLT-C, (8.7 kbp) | *Polyhedrin* | ATG, GST, *Bam*H I, 6× His tag-Protein kinase A site, Thrombin cleavage, *Nde* I, *Xho* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | | | |
| | pAcGP67-A (9.8 kbp) | *Polyhedrin* | ATG, GP64 signal sequence, *Bam*H I, *Sma* I, *Xba* I, *Eco*R I, *Not* I, *Eag* I, *Pst* I, *Bgl* II, *Ppum* I | A, B, and C represent three different reading frames and also contain slightly different restriction sites. | | |
| | pAcGP67-B (9.8 kbp) | *Polyhedrin* | ATG, GP64 signal sequence, *Bam*H I, *Sma* I, *Nco* I, *Eco*R I, *Not* I, *Eag* I, *Pst* I, *Bgl* II | | | |
| | pAcGP67-C (9.8 kbp) | *Polyhedrin* | ATG, GP64 signal sequence, *Bam*H I, *Sma* I, *Nco* I, *Eco*R I, *Not* I, *Eag* I, *Pst* I, *Bgl* II, *Ppum* I | | | |
| | pAcHLT-A (8.1 kbp) | *Polyhedrin* | ATG, GST, *Bam*H I, 6× His tag, Protein kinase A site-Thrombin cleavage, *Nde* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | A, B, and C represent three different reading frames and also contain slightly different restriction sites. | | |
| | pAcHLT-B (8.1 kbp) | *Polyhedrin* | ATG, GST, *Bam*H I, 6× His tag, Protein kinase A site, Thrombin cleavage-*Xho* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | | | |
| | pAcHLT-C (8.1 kbp) | *Polyhedrin* | ATG, GST, 6× His tag, Protein kinase A site, Thrombin cleavage site, *Nde* I, *Xho* I, *Eco*R I, *Stu* I, *Nco* I, *Sac* I, *Not* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | | | |

| | | | | | |
|---|---|---|---|---|---|
| | pAcSecG2T (8.6 kbp) | *Polyhedrin* | ATG, GP64 signal sequence, GST, Thrombin cleavage site, *Bam*H I, *Sma* I, *Eco*R I | | |
| | pAcSG2 (5.5 kbp) | *Polyhedrin* | *Xho* I, *Eco*R I, *Stu* I, *Nco* I, ATG, *Sac* I, *Not* I, *Eag* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | ATG codon for fusion proteins | |
| Single promoter (*Polyhedrin*) | pAcMP2 (9.8 kbp) | *Basic* (*p6.9*) | *Pst* I, *Not* I, *Eag* I, *Eco*R I, *Xba* I, *Bam*H I - *polyhedrin* PA | *p*6.9 promoter provides late gene expression | |
| | pAcMP3 (9.8 kbp) | | *Bam*H I, *Xba* I, *Eco*R I, *Not* I, *Eag* I, *Pst* I, *Bgl* II | | |
| Multiple promoter (*Polyhedrin*) | pAcUW51 (5.8 kbp) | *Polyhedrin*<br>*p10* | *Bam*H I<br>*Bgl* II, *Eco*R I | Dual expression vector from polyhedrin and p10 promoters | |
| | pAcAB4 (10.2 kbp) | *Polyhedrin*<br>*P10*<br>*Polyhedrin*<br>*p10* | *BamHI I*<br>*Sma* I<br>*Xba* I, *Stu* I<br>*Bgl* II, *Eco*R I, *Esp* I | Quadruple expression vector from 2×polyhedrin and 2×p10 promoters | |
| Single promoter (*Polyhedrin*) | pOET1 (4.5 kbp) | *Polyhedrin* | *Bam*H I, *Hin*d III, *Xba* I, *Sal* I, *Apa* I, *Xma* I, *Sma* I, *Pst* I, *Eco*R I, *Sac* I, *Xho* I, *Asp*718I, *Kpn* I, *Bse* X31, *Eag* I, *Not* I, *Sac* II, *Bgl* II, *Pac* I | | Oxford Expression Technologies |
| | pOET1N_6× His (4.6 kbp) | *Polyhedrin* | ATG, 6× His tag, *Age* I, thrombin cleavage, MCS as pOET1 | | |
| | LICpOET1N_His (6.8 kbp) | *Polyhedrin* | *Bam*H I, *Hin*d III, *Sma* I, *Nco* I, ATG, 6× His tag, *Bse*R I (LIC 5′ site), TEV cleavage, SacB, *Bse*R I (LIC3 site), *Bam*H I, *Eco*R I, *Hin*d III | Includes SacB gene, for negative selection of bacteria in 5 % sucrose | |

(continued)

**Table 1**
**(continued)**

| Type (*locus*) | Vector (size) | Promoter(s) | Features within cloning sites and poly(A) signal (PA) | Additional features/ notes | Source | Parental virus (Source) |
|---|---|---|---|---|---|---|
| | LIC_pOET1N_His_ GP64 (6.8 kbp) | *Polyhedrin* | *Bam*H I, *Hin*d III, i I, *Nco* I, ATG, GP64 signal peptide, 6× His tag, TEV cleavage, *Bse*R I (LIC 5′ site), SacB, *Bse*R I (LIC3 site), *Bam*H I, *Eco*R I, *Hin*d III | | | |
| | pOET1C_6× His (4.6 kbp) | *Polyhedrin* | MCS (*Bam*H I – *Bgl* II) as pOET1, 6× His tag, *Pac*I | | | |
| | pOET2 (4.5 kbp) | *Polyhedrin* | *Bgl* II, *Bse*X 31, *Eag* I, *Not* I, *Sac* II, *Asp* 718I, *Kpn* I, *Xho* I, *Sac* I, *Eco*R I, *Pst* I, *Xma* I, *Sma* I, *Apa* I, *Sal* I, *Xba* I, *Hin*d III, *Bam*H I, *Pac* I | | | |
| | pOET2N/C_6× His (4.6 kbp) | *Polyhedrin* | ATG, 6× His tag, thrombin cleavage, MCS as pOET2 (*Bgl* II-*Bam*H I), 6× His tag, *Spe* I, *Pac* I | | | |
| | pOET2C_6× His (4.6 kbp) | *Polyhedrin* | MCS as pOET2 (*Bgl* II – *Bam*H I, *Age* I, 6× His tag, *Pac* I | | | |
| | pOET3 (4.5 kbp) | *p6.9* | *Bam*H I, *Hin*d III, *Xba* I, *Sal* I, *Apa* I, *Xma* I, *Sma* I, *Pst* I, *Eco*R I, *Sac* I, *Xho* I, *Asp* 718I, *Kpn* I, *Bse*X 31, *Eag* I, *Not* I, *Sac* II, *Bg l*II, *Pac* I | | | |
| | LICpOET3N_His (6.7 kbp) | *p6.9* | *Bam*H I, *Hin*d III, *Sma* I, *Nco* I, ATG, 6× His tag, *Bse*R I (LIC 5′ site), TEV cleavage, SacB, *Bse*R I (LIC3 site), *Bam*H I, *Eco*R I, *Hin*d III | | | |
| | LIC_pOET3N_His_ GP64 (6.8 kbp) | *p6.9* | *Bam*H I, *Hin*d III, *Sma* I, *Nco* I, ATG, GP64 signal peptide, 6× His tag, TEV cleavage, *Bse*R I (LIC 5′ site), SacB, *Bse*R I (LIC3 site), *Bam*H I, *Eco*R I, *Hin*d III | | | |
| | pOET4 (4.5 kbp) | *p6.9* | *Bgl* II, *Bse*X 31, *Eag* I, *Not* I, *Sac* II, *Asp* 718I, *Kpn* I, *Xho* I, *Sac* I, *Eco*R I, *Pst* I, *Xma* I, *Sma* I, *Apa* I, *Sal* I, *Xba* I, *Hin*d III, *Bam*H I, *Pac* I | | | |

| | | | | | |
|---|---|---|---|---|---|
| Multiple promoter (*Polyhedrin*) | pOET5 | *Polyhedrin P10* | *Bam*H I, *Sal* I, *Xho* I, *Sma* I, *Kpn* I, *Hin*d III *Bgl* II, *Eco*R I, *Sac* I, *Stu* I, *Not* I | Dual expression vector from polyhedrin and p10 promoters | |
| Single promoter (*Polyhedrin*) | pPolh-FLAG™-1 (5.6 kbp) | *Polyhedrin* | ATG, FLAG tag, enterokinase cleavage, *Xho* I, *Not* I, *Eco*R I, *Pst* I, *Sma* I, *Kpn* I, *Bam*H I, *Bgl* II | | Sigma-Aldrich |
| | pPolh-FLAG™-2 (5.6 kbp) | *Polyhedrin* | *Xho* I, *Not* I, *Eco*R I, *Pst* I, *Sma* I, *Kpn* I, *Bam*H I, *Bgl* II, FLAG tag, stop | | |
| | pPolh-MAT™-1 (5.6 kbp) | *Polyhedrin* | ATG, metal affinity tag (MAT), *Xho* I, *Not* I, *Eco*R I, *Pst* I, *Sma* I, *Kpn* I, *Bam*H I, *Bgl* II | | |
| | pPolh- MAT™-2 (5.6 kbp) | *Polyhedrin* | *Xho* I, *Not* I, *Eco*R I, *Pst* I, *Sma* I, *Kpn* I, *Bam*H I, *Bgl* II, MAT, stop | | |
| | pAB-bee™ (9.9 kbp) | *Polyhedrin* | ATG, honeybee melittin signal, *Not* I, *Xba* I, *Sma* I, *Bam*H I, MCS stuffer, *Eco*R I, 8× His tag, stop, *Avr* II, *Sal* I | | AB vector |
| | pAB-bee™-8× His (9.9 kbp) | *Polyhedrin* | ATG, honeybee melittin signal, 8× His tag, *Not* I, *Eco*R I, *Xba* I, *Sma* I, *Bam*H I, MCS stuffer, *Eco*R I, 8× His tag, stop | | |
| | pAB-bee-FH (9.8 kbp) | *Polyhedrin* | ATG, honeybee melittin signal, *Not* I, *Eco*R I, *Sma* I, *Bam*H I, *Xba* I, FLAG tag, 8× His tag, stop | | |
| | pAB-6× His™ (9.7 kbp) | *Polyhedrin* | ATG, 6× His tag, *Spe* I, thrombin cleavage, *Sma* I, *Xba* I, *Eco*R I, *Not* I, *Pst* I, *Bgl* II | | |
| | pAB-6× His-MBP™ (10.8 kbp) | *Polyhedrin* | ATG, 6× His tag, *Spe* I, Maltose binding protein, *Sac* I, *Stu* I, thrombin cleavage, *Xba* I, *Eco*R I, *Sma* I, *Not* I | | |
| | pVL-FH (9.7 kbp) | *Polyhedrin* | *Bgl* II, *Pst* I, *Not* I, *Eco*R I, *Sma* I, *Bam*H I, *Xba* I, FLAG tag, 8× His tag, stop | | |

(continued)

**Table 1**
**(continued)**

| Type (*locus*) | Vector (size) | Promoter(s) | Features within cloning sites and poly(A) signal (PA) | Additional features/ notes | Source | Parental virus (Source) |
|---|---|---|---|---|---|---|
| | pVL-GFP (10.4 kbp) | *Polyhedrin* | *Bam*H I, *Sma* I, *Xba* I, *Eco*R I, *Not* I, *Pst* I, GFP | For fusing proteins with C-terminal GFP | | |
| | pAB-GST™ (10.4 kbp) | *Polyhedrin* | ATG, GST, PreScission™ cleavage, *Bam*H I, *Eco*R I, *Sma* I, *Not* I, *Pst* I, *Bgl* II | | | |
| | pAB-MBP™ (10.8 kbp) | *Polyhedrin* | ATG, maltose binding protein, *Sac* I, *Stu* I, thrombin cleavage, *Xba* I, *Eco*R I, *Sma* I, *Not* I | | | |
| | pAB-6× His-MBP™ (10.8 kbp) | *Polyhedrin* | ATG, 6× His tag, *Spe* I, maltose binding protein, *Sac* I, *Stu* I, thrombin cleavage, *Xba* I, *Eco*R I, *Sma* I, *Not* I | | | |
| | pAcAB3 (10.1 kbp) | *P10*<br>*Polyhedrin*<br>*P10* | *Bgl* II, *Esp* I<br>*Xba* I, *Stu* I<br>*Sma* I, *Bam*H I | | | |
| | pAcIRES (5.8 kbp) | *Polyhedrin* | *Xho* I, *Eco*R I, IRES, *Nco* I, *Sty* I, *Ban* II, *Sac* I, *Not* I, *Eag* I, *Sse*8387 I, *Pst* I, *Kpn* I, *Sma* I, *Bgl* II | Internal ribosome entry site (IRES) from Cricket paralysis virus for translation of two ORFs from single mRNA | | |

| | | | | | |
|---|---|---|---|---|---|
| Single promoter (*Polyhedrin*) | pBAC-1 (5.3 kbp) | *Polyhedrin* | *Bam*H I, *Stu* I, *Eco*R I, *Sac* I, *Hin*d III, *Eag* I, *Not* I, *Ava* I, *Xho* I, 6× His tag, *Sty* I, *Avr* II, *Bpu* 1102, *Sph* I | | EMD Millipore |
| | pBAC-3 (5.5 kbp) | *Polyhedrin* | Gp64 signal peptide, *Nco* I, 6× His tag, *Sac* II, thrombin cleavage site, S tag, *Pfl*M I, *Nhe* I, enterokinase cleavage site, *Sma* I, *Srf* I, *Bse*R I, *Stu* I, *Bam*H I, *Eco*R I, *Sac* I, *Hin*d III, *Eag* I, *Not* I, *Xho* I, 6× His tag, *Avr* II, *Bpu*1102 I, *Sph* I | | |
| | pTriEx™-1.1 | *p10* | *Nco* I, *Eco*R V, *Sma* I, *Sac* I, *Bam*H I, *Eco*R I, *Bgl* II, *Asc* I, *Pst* I, *Kpn* I, *Nsp* V, *Hin*d III, *Not* I, *Pvu* II, *Pml* I, HSV tag, *Xho* I, 6× His tag, *Dra* III – rabbit ß globin PA | Also has CMV ie enhancer, chicken ß actin promoter and T7lac promoter for expression in mammalian and bacterial cells | |
| | pTriEx™-2 | *p10* | *Nco* I, 6× His tag, *Xcm* I, S tag, thrombin cleavage, *Sma* I, enterokinase cleavage, *Psh*AI, *Bse*R I, *Eco*R V, *Sac* I, *Bam*H I, *Eco*R I, *Bgl* II, *Asc* I, *Pst* I, *Kpn* I, *Pin*A I, *Nsp* V, *Pvu* II, *Pml* I, HSV tag, *Xho* I, 6× His tag, *Dra* III – rabbit ß globin PA | Other features as pTriEx™-1.1 | |
| | pTriEx™-3 | *p10* | *Nco* I, *Eco*R V, *Sma* I, *Sac* I, *Bam*H I, *Bg lI*I, *Asc* I, *Pst* I, *Kpn* I, *Pin*A I, *Nsp* V, *Hin*d III, *Not* I, *Pvu* II, *Bst* 1107, *Pml* I, HSV tag, *Xho* I, 6× His tag, *Dra* III | CMV ie enhancer/ promoter and T7lac promoter for expression in mammalian and bacterial cells | |

(continued)

**Table 1**
**(continued)**

| Type (*locus*) | Vector (size) | Promoter(s) | Features within cloning sites and poly(A) signal (PA) | Additional features/ notes | Source | Parental virus (Source) |
|---|---|---|---|---|---|---|
| | pTriEx™-4 | *p10* | *Nco* I, 6× His tag, *Xcm* I, S tag, *Sma* I, thrombin cleavage, enterokinase cleavage, *Psh*A I, LIC site, *Bse*R I, *Eco*R V, *Sac* I, *Bam*H I, *Eco*R I, *Bgl* II, *Asc I*, *Pst* I, *Kpn* I, *Pin*A I, *Nsp* V, *Hin*d III, *Not* I, *Pvu* II, *Pst* 1107, *Pml* I, HSV tag, *Xho* I, 6× His tag, *Dra* III | Other features as pTriEx™-3. Also includes LIC cloning site (*Bse*RI) | | |
| Multiple promoter (*polyhedrin*) | pBAC4×-1 (5.9 kbp) | *p10*<br>*Polyhedrin*<br>*p10*<br>*Polyhedrin* | *Bgl* II, *Eco*R I, *Bsu*36 I<br>*Xba* I, *Stu* I<br>*Sma* I, *Spe* I<br>*Bam*H I, *Hin*d III, *Eag* I, *Not* I, *Xho* I 6× His tag, *Sty* I, *Bpu*1102 I, *Sph* I | Quadruple expression vector from 2×polyhedrin and 2×p10 promoters. | | |
| Single promoter (*polyhedrin*) | pBAC-5 (5.5 kbp) | *Gp64* | *Nco* I, 6× His tag, *Sac* II, Thrombin cleavage site, S tag, *Pfl*M I, *Nhe* I, enterokinase cleavage site, *Sma* I, *Srf* I, *Bse*R I, *Stu* I, *Bam*H I, *Eco*R I, *Sac* I, *Hin*d III, *Eag* I, *Not* I, *Xho* I, 6× His tag, *Avr* II, *Bpu*1102 I, *Sph* I.<br>within S tag. | Has early/late *gp64* promoter but no signal peptide coding region. | | |
| | pBAC-6 (5.6 kbp) | *Gp64* | GP64 signal sequence, *Nco* I, 6× His tag, *Sac* II, Thrombin cleavage site, S tag, *Pfl*M I, *Nhe* I, enterokinase cleavage site, *Sma* I, *Srf* I, *Bse*R I, *Stu* I, *Bam*H I, *Eco*R I, *Sac* I, *Hin*d III, *Eag* I, *Not* I, *Xho* I, 6× His tag, *Avr* II, *Bpu*1102 I, *Sph* I.<br>within S tag. | Has early/late gp64 promoter and signal peptide coding region. | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | pBACsurf-1 (9.4 kbp) | *Polyhedrin* | *Spe* I, gp64 signal sequence, *Pst* I, *Kpn* I, *Sma* I, gp64 coding region | Designed for incorporating target proteins on the virion surface by utilizing gp64 signal sequence and membrane anchor region. | | |
| Multiple promoter (*polyhedrin*) | pUCDM (3.0 kbp) | *Polyhedrin*<br><br>*p10* | *Bam*H I, *Rsr* II, *Bss*H II, *Stu* I, *Sal* I, *Sac* I, *Xba* I, *Pst* I<br>*Bbs* I, *Sma* I, *Xma* I, *Xho* I, *Nhe* I, *Nsi* I, *Sph* I | *Bst*Z 171, *Spe* I, *Cla* I, *Nru* I sites between pol and p10 promoters for insertion of expression modules from pFBDM or reinsertion in this vector. Pol and p10 multicloning sites are flanked by *Avr* II and *Pme* I sites respectively. | [34] | DH10BMultiBac*cre* maintained in *E. coli*. Contains Tn7 site at the polyhedrin locus and Cre-loxP site in lieu of chitinase/cathepsin genes for simultaneous integration of foreign genes. Cre-loxP integration is mediated by pBADZ-His$^6$Cre under arabinose control [34]. |
| Multiple promoter (*chitinase/ cathepsin*) | pFBDM (5.3 kbp) | *Polyhedrin p10*<br><br>*p10* | *Bam*H I, *Rsr* II, *Bss*H II, *Eco*R I, *Stu* I, *Sal* I, *Sac* I, *Not* I, *Bst*B I, *Xba* I, *Pst* I, *Hin*d III<br>*Bbs* I, *Sma* I, *Xma* I, *Xho* I, *Nhe* I, *Nsi* I, *Sph* I, *Kpn* I | Polyhedrin and p10 multicloning sites are flanked by *Avr* II and *Pme* I sites respectively. | | |
| Single promoter (*p10*) | pAcUW1 (4.6 kbp) | *p10* | *Bgl* II, *Hin*d III | | [30] | AcUW1.lacZ virus DNA linearized with *Bsu*36 I [30] |

### 1.2.2 Linear Virus DNA

The proportion of recombinant virus plaques that could be isolated from a co-transfection rose to about 30 % when it was discovered that linear baculovirus DNA had much reduced infectivity compared with its circular counterpart [12]. Linearization was achieved by replacing the polyhedrin gene with a *Bsu*36 I restriction enzyme site, which is normally absent from the virus genome. Although the parental virus DNA used in this approach was polyhedrin gene negative, plaques could be isolated at random in the knowledge that a good proportion of them would contain the required foreign gene. This system was developed further by inserting the coding region for bacterial β-galactosidase into the AcMNPV genome under the control of the polyhedrin gene promoter.

The AcMNPV genome was also modified to contain *Bsu*36 I sites on both sides of the β-galactosidase sequences, so that digestion with this enzyme removed the gene and also part of a virus gene (ORF 1629) that encodes a structural protein [13]. By removing part of the essential ORF 1629 gene, the virus is unable to form infectious particles efficiently even if the linear DNA is recircularized in insect cells. Instead, a process of homologous recombination repairs the deletion in ORF 1629 while simultaneously inserting the foreign gene in place of β-galactosidase. Recombinant plaques comprise nearly 100 % of the progeny virus population. Those few parental plaques stain blue in the presence of X-gal and are easily avoided in titrations.

Some parental virus genomes have been modified further to delete some of the genes that are not essential for replication in cell culture. These genes include chitinase [14] and cathepsin [15, 16] that have been associated with degradation of recombinant proteins on prolonged exposure in cell culture [17]. Removal of both chitinase and cathepsin from a recombinant virus greatly improves expression yields of some proteins [18].

Although the use of AcMNPV DNA digested with *Bsu*36 I promotes efficient recovery of recombinant viruses, there remain a very low level of parental viruses contaminating the progeny virus stocks [13]. These have to be removed by titration with a plaque assay in insect cells. The requirement for this step makes it very difficult to scale up procedures for high throughput production of recombinant viruses. Over digestion of parental virus DNA does not eliminate every circular genome. This problem has been addressed by inserting the virus genome, lacking part of ORF1629, into a low copy number plasmid vector and amplifying the DNA in *E. coli* [19, 20]. This DNA is unable to initiate an infection after transfection of insect cells, unless it is rescued by a transfer vector containing the complete ORF1629 and a foreign gene. This ensures 100 % recovery of recombinant viruses without the need to perform plaque purification. The system is also amenable to parallel processing of many recombinant viruses using manual or auto-

mated methods. The virus DNA is now marketed as *flash*Bac™ by Oxford Expression Technologies.

### 1.2.3 Virus DNA Within Bacterial Hosts

A radical departure in techniques for deriving recombinant baculoviruses came with the introduction of "bacmid" technology [21]. A modified baculovirus genome was derived containing a mini-F replicon, selectable marker and a Tn7 transposition site. This large plasmid-baculovirus molecule can be maintained at low copy number in *E. coli*. A helper plasmid encoding the Tn7 transposase functions is also introduced into these cells so that transposition of sequences can be used to insert a foreign coding region into the baculovirus genome. The baculovirus transfer vector used for this purpose differs from earlier designs. The plasmid contains the desired foreign coding region and a second selectable marker for bacterial cells between the left and right arms of Tn7. The transposition functions provided by the helper plasmid achieves removal of the coding region and marker from the transfer vector to the bacmid, which is selected on appropriate agar plates. The DNA recovered from these amplified colonies is used to transfect insect cells and derive infectious virus particles for subsequent analysis. This system (Bac-to-Bac, Life Technologies) is simple and appeals to those used to prokaryotic molecular biology.

### 1.2.4 In Vitro Manipulation of Baculovirus DNA

Earlier methods for manipulating baculovirus DNA in vitro did not become widely adopted [5–7], but recent developments have resulted in a commercially available system for rapid assembly of recombinant virus genomes and their subsequent introduction into insect cells.

Ganciclovir is a nucleoside analog (9-[(1,3-Dihydroxy-2-propoxy)methyl] guanine) that is phosphorylated by the product of a Herpes Simplex Virus type 1 (HSV1) thymidine kinase (*tk*) gene. After phosphorylation, the active analog incorporates into DNA and inhibits DNA replication [22]. Baculovirus expression of *tk* in insect cells makes the virus sensitive to ganciclovir [23]. It forms the basis of a negative selection system for the production of recombinant baculoviruses.

The BaculoDirect™ baculovirus expression system includes *att*R sites for recombination with a Gateway entry clone. These sites flank the HSV1-*tk*, which is under the control of the AcMNPV *ie-0* promoter and the β-galactosidase coding region under the control of the *p10* promoter. Parental BaculoDirect™ DNA is sensitive to ganciclovir. A foreign gene is transferred from the entry clone into the BaculoDirect™ linear DNA using a 1 hour enzymatic reaction. The correct introduction of the foreign gene into the virus should be accompanied by removal of a β-galactosidase coding region and the HSV1-*tk*, which then renders this modified

virus insensitive to ganciclovir. This recombinant virus genome is then introduced into insect cells via transfection to derive infectious virus particles. To ensure that the derived virus stock is free of parental virus material, the transfected cells are incubated with ganciclovir and stained for β-galactosidase production. Unlike the bacmid system described above, there is no need to use an intermediate bacterial host.

With this system the role of the transfer vector is subtly different from the early technologies. The Gateway® entry vectors permit cloning of PCR products that can then be transferred in vitro to any of the other compatible plasmids. The requirement for homologous recombination in insect cells between virus and transfer vector is removed. The introduction of genes into the virus is accomplished in vitro, with subsequent transfection of insect cells and of recombinant virus in an antibiotic-containing medium. It means that several viruses can be readily processed simultaneously. However, flexibility in the system is reduced as modifications to promoters and other features, e.g., fusions with signal peptide sequences and peptide tags, become harder to perform. Currently, one is limited to three BaculoDirect™ constructs, i.e., (1) N-terminal tagging of a protein with V5-His tags, (2) C-terminal tagging of a protein with V5-His tags, and (3) a combination of N-terminal V5-His tagging and a C-terminal honeybee melittin secretion signal to enhance protein secretion. Note that the V5-His tags greatly simplify protein purification as described in Chapter 1.

## 2 Transfer Vectors for Expression of Single Genes

### 2.1 Polyhedrin Gene Locus Expression Vectors

The first transfer vectors to be developed for baculovirus expression systems were based on the polyhedrin gene promoter. This is a very late gene promoter active in the latter stages of virus infection when occlusion bodies or polyhedra are being assembled. It requires the presence of a virus-encoded alpha amanitin RNA polymerase for activation. Its primary structure has been elucidated and has been reviewed [24]. Essentially, it comprises a TAAG core site where transcription initiates flanked by an upstream 20 nucleotide region and a 50 nucleotide downstream 5′ noncoding region that are required for optimal gene expression. Prior to the work that characterized the promoter, some early baculovirus transfer vectors contained a truncated 5′ noncoding region that was suboptimal for very late gene expression. All vectors used currently have the complete 5′ noncoding region, which appears to contain a burst sequence just before the ATG of the native polyhedrin. A list of the vectors most commonly used today is provided in Table 1, together with the virus DNA compatible with them. A feature of many of these vectors is the presence of multiple restriction enzyme sites for

inserted foreign sequence with variable ends. Many of the vectors also have other features, such as histidine tags or protease cleavage sites to enable post-expression protein purification.

### 2.2 Transfer Vectors Utilizing Alternative Gene Promoters

Theoretically, a copy of any baculovirus gene promoter could be used for expression of foreign genes. In practice, only a handful of promoters other than the polyhedrin or p10 have been used for recombinant protein production. Most of these are active in the late phase of baculovirus gene expression, between about 8 and 24 h post-infection. The polyhedrin gene promoter was replaced with the basic protein or p6.9 gene promoter in pAcMP1 [25]. This vector has been modified further to derive pAcMP2 and 3, which have multicloning sites and are supplied by BD Biosciences (Table 1). A similar vector was produced that utilized the p39 capsid gene promoter [26], but this is not marketed commercially. The advantage of using late gene promoter vectors is that recombinant proteins are made in a phase of virus replication when the virus itself is assembling complex structures and has to produce glycoproteins. It is reasonable to assume that if recombinant proteins require substantial post translational modifications that these will be performed better in the late phase of gene expression. Additional details of post-translational modifications are given in Chapter 18. The glycoprotein (GP64/67) specific for the budded form of the baculovirus is under the control of a hybrid early/late gene promoter [27]. This promoter is included in some transfer vectors, e.g., pBAC-5 and -6, for earlier expression of recombinant proteins (Table 1).

### 2.3 p10 GeneLocus-Based Vectors

The p10 gene locus offers an alternative to the use of the polyhedrin gene region for the insertion of foreign genes. The p10 gene promoter offers slightly earlier activation in comparison to the polyhedrin gene promoter [28], but overall total gene expression may be slightly lower [29]. The main problem in using the p10 gene locus is that there are fewer selection systems that have been developed to make it easy to insert foreign genes. An AcMNPV variant, AcUW1.lacZ, was developed so that its DNA can be linearized within the lacZ sequences using *Bsu*36I [30]. Analogous to the use of linearization at the polyhedrin gene locus [12, 13], recovery of recombinant viruses is close to 30 % efficient when this linear DNA is used in combination with the appropriate plasmid transfer vector [30]. The transfer vector pAcUW1 contains a copy of the complete *p10* promoter, which is required for efficient expression of foreign genes [31, 32]. Only a single restriction enzyme site, *Bgl*II, was inserted in lieu of the p10 ATG and 5′ end of the coding region, but a *Hin*dIII site in the remainder of the p10 coding region offers the potential for cloning foreign DNA with asymmetric ends.

## 3 Transfer Vectors for Multiple Gene Expression

### 3.1 Polyhedrin Gene Locus

The polyhedrin gene locus has been employed most widely as a location for assembling multigene constructs. Table 1 lists many vectors that are currently available. The first dual expression vector (pAcVC2) utilized two polyhedrin gene promoters [33]. Recent developments tend to use both p10 and polyhedrin gene promoters, with two copies of each permitting quadruple expression of recombinant genes at the polyhedrin gene locus (e.g., pAcAB4; BD Biosciences; Table 1). The use of such vectors requires careful planning of the order in which genes are inserted, as subsequent digestion of the transfer vector at each stage may lead to cleavage of an earlier foreign gene coding region with a restriction enzyme. Most of these vectors have a range of restriction sites available at each promoter to facilitate foreign gene insertion.

An improved system for the production of multiprotein complexes using baculoviruses and insect cells uses transfer vectors (pUCDM and pFBDM) containing a multiplication module with polyhedrin and p10 gene promoters, which can be nested to enable assembly of polycistronic expression cassettes [34]. The pUCDM vector is used to insert foreign genes at the polyhedrin gene locus via Tn*7* transposition within an *E. coli* host. The pFBDM vector inserts multigene modules at the *chitinase/cathepsin* locus [14, 15] via Cre-loxP site-specific recombination in bacterial cells containing the Cre recombinase. Deletion of the cathepsin gene was also reported to reduce recombinant protein degradation in virus-infected insect cells [34].

### 3.2 p10 Gene Locus

Despite its attraction as a site for inserting foreign genes into the baculovirus genome, the p10 gene locus remains undeveloped for this purpose. This is probably a consequence of the lack of an easy selection method for recombinant viruses. The two vectors previously available for dual gene expression at the *p10* locus, pAcUW42, and 43 were developments of pAcUW1. They have a copy of the polyhedrin gene promoter inserted downstream of the native p10 promoter so that two genes can be expressed. Simian virus 40 polyadenylation signals were inserted between the two promoters to terminate transcripts from the *p10* promoter. Unfortunately, these vectors are no longer commercially available, although an earlier version (pAcUW41) can be obtained from the authors.

## 4 Transfer Vectors for Secretion of Recombinant Proteins or Surface Display on the Virus

Baculovirus transfer vectors have been produced that contain signal peptide coding regions for directing recombinant proteins into the endoplasmic reticulum of the virus-infected cell. Most utilize

the *gp64/67* signal peptide (e.g., pBAC-3 or pBAC-6 from EMD Millipore). The pBAC-3 vector uses the polyhedrin gene promoter but pBAC-6 employs the native *gp64/67* promoter, which directs foreign gene expression in both early and late phases. Invitrogen markets pFastBac™ HBN-TOPO, which contains the honeybee melittin secretion signal [35] and should be used with the Bac-to-Bac system for making recombinant viruses [35]. Table 1 contains details of several other vectors suitable for the secretion of recombinant proteins with or without peptide tags. Surface display of recombinant proteins on the budded virus particles is feasible if pBACsurf-1 (EMD Millipore) is used. This has the *gp64/67* promoter, signal sequence and anchor coding sequence from the carboxyl terminal of the protein. Recombinant proteins are inserted into the plasma membrane of virus-infected cells and then as nucleocapsids bud through during virus maturation are incorporated into the virus envelope.

## 5 Ligation Independent Cloning of Genes Into Transfer Vectors

It is now increasingly common for baculovirus transfer vectors to have the facility for ligation independent cloning (LIC) incorporated into their design. This method involves the generation of compatible sticky ends in the restriction enzyme-digested plasmid and a DNA fragment specifying the target gene by incubation with T4 DNA polymerase [36, 37]. The annealing of these two molecules in a matter of minutes results in a circular molecule, which can be used to transform bacteria. The use of PCR to generate the gene coding sequence also renders the method amenable to scale up to a high throughput process utilizing 96-well plates.

## 6 Summary

With the availability of many baculovirus expression systems on a commercial basis, many of the problems previously encountered by new users of the technology can be avoided. It is now possible to select appropriate baculovirus transfer vectors according to the requirements of a particular project and with regard to the previous expertise of the user. Those familiar with prokaryotic expression may be more comfortable using the Bac-to-Bac system, which assembles recombinant viruses in *E. coli*, prior to recovery of infectious virus DNA for transfection of insect cells. Those users with more experience in eukaryotic systems may prefer to use traditional co-transfection of insect cells with virus DNA and plasmid transfer vectors to exploit the wider range of reagents available for this approach. High throughput production of recombinant viruses is now feasible given the introduction of BaculoDirect™, which allows

recombinant virus DNA assembly in vitro, and the *flash*Bac™ system, which utilizes a one-step co-transfection step in insect cells that can be automated. Transfer vectors with LIC features also greatly simplify the insertion of foreign sequences. One of the major advantages of the baculovirus system is that it is amenable to very different scales of activity. These now range from single recombinant virus production to parallel processing of hundreds of samples.

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# Chapter 4

# Recombinant Baculovirus Isolation

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## Abstract

Although there are several different methods available of making recombinant baculovirus expression vectors (reviewed in Chapter 3), all require a stage in which insect cells are transfected with either the virus genome alone (Bac-to-Bac® or BaculoDirect™, Invitrogen) or virus genome and transfer vector. In the latter case, this allows the natural process of homologous recombination to transfer the foreign gene, under control of the polyhedrin or other baculovirus gene promoter, from the transfer vector to the virus genome to create the recombinant virus. Previously, many methods required a plaque-assay to separate parental and recombinant virus prior to amplification and use of the recombinant virus. Fortunately, this step is no longer required for most systems currently available. This chapter provides an overview of the historical development of increasingly more efficient systems for the isolation of recombinant baculoviruses (Chapter 3 provides a full account of the different systems and transfer vectors available). The practical details cover: transfection of insect cells with either virus DNA or virus DNA and plasmid transfer vector; a reliable plaque-assay method that can be used to separate recombinant virus from parental (non-recombinant) virus where this is necessary; methods for the small-scale amplification of recombinant virus; and subsequent titration by plaque-assay or real-time polymerase chain reaction (PCR). Methods unique to the Bac-to-Bac® system are also covered and include the transformation of bacterial cells and isolation of bacmid DNA ready for transfection of insect cells.

**Key words** Recombinant virus isolation, Co-transfection, Insect cells, Plaque-assay, Virus amplification

## 1 Introduction

The baculovirus genome is generally considered too large to insert the foreign gene by direct ligation, although one commercial expression system (BaculoDirect™, Life Technologies) now utilizes Gateway® recombination technology to insert a gene directly into linearized virus DNA. With most systems, however, the foreign gene is cloned into a plasmid, usually referred to as the transfer vector, which contains sequences that flank the polyhedrin gene in the virus genome. The parental virus genome and the transfer vector are introduced into the host insect cell and homologous recombination, between the flanking sequences common to both DNA molecules, mediates insertion of the foreign gene

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into the virus DNA, resulting in a recombinant virus genome. The genome then replicates to produce recombinant budded virus without occlusion body formation as the polyhedrin gene is no longer functional. This can be harvested from the culture medium for further propagation and analysis of foreign gene expression. This process was first reported by Smith et al. [1] in which they described the production of a recombinant virus expressing the β-interferon gene.

In most baculovirus expression systems that use homologous recombination to transfer the foreign gene into the virus genome, a mixture of recombinant and original parental virus is produced after the initial round of replication. Before using the virus as an expression vector, the recombinant virus has to be separated from the parental virus. Traditionally, this has been achieved by plaque-assay and plaque-purification.

Several attempts have been made to improve the methods by which recombinant and parental virus may be separated. The frequency of recombination using the original system is low (<1 %) and thus recombinant virus plaques can be obscured by an excess of parental virus plaques. This problem was partially addressed by the insertion of the *Escherichia coli lacZ* gene into the virus genome, in addition to the gene of interest. The recombinant virus plaques could then be stained blue by the addition of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) against a background of colorless parent plaques. However, this did not improve the low recombination efficiency and resulted in the production not only of the recombinant protein but also β-galactosidase [2].

The efficiency with which recombinant virus could be recovered was improved by the addition of a unique restriction enzyme site (*Bsu*36I) at the polyhedrin locus (AcRP6-SC). Linearization of the virus genome prior to homologous recombination reduced the infectivity of the virus DNA but increased the proportion of recombinant virus recovered to 30 % [3]. Homologous recombination between the transfer vector and the linear DNA re-circularized the virus genome, restoring infectivity and the production of virus particles. *LacZ* was then introduced at the polyhedrin gene locus, replacing the polyhedrin gene coding region, producing AcRP23.*lacZ*. A *Bsu*36I restriction site within *lacZ* allowed for more efficient restriction of the linear DNA prior to homologous recombination and the presence of *lacZ* allowed the selection of colorless recombinant virus plaques against a background of blue parental virus plaques in the presence of X-gal [3].

Greater than 90 % recovery of recombinant virus plaques was achieved by further modifications to produce BacPAK6 [4]. BacPAK6 contains the *E. coli lacZ* gene inserted at the polyhedrin gene locus and *Bsu*36I restriction enzyme sites in two flanking genes on either side of *lacZ*. Digestion with *Bsu*36I removes the *lacZ* gene and a fragment of an essential gene (ORF 1629) [5]

producing linear BacPAK6 virus DNA that is unable to replicate within insect cells. Co-transfection of insect cells with BacPAK6 DNA and a transfer vector containing the gene of interest under the control of the polyhedrin gene promoter restores ORF 1629 and re-circularizes the virus DNA by allelic replacement. The recombinant baculovirus DNA is then able to replicate in insect cells, virions are assembled and recombinant baculoviruses are produced in the late phase of infection. However, *Bsu*36I digestion is never 100 % efficient and the final virus population will always contain a mixture of recombinant and parental virus that requires purification by plaque-assay [4].

Further developments have described baculovirus expression systems that do not require separation and purification of recombinant viruses by plaque-assay. The first of these to be described, the so-called Bac-to-Bac® system (Life Technologies), is now widely used in many laboratories [6–8]. The Bac-to-Bac® system is based upon site-specific transposition of the foreign gene to be expressed from a plasmid vector into a baculovirus genome, maintained as a bacmid in *E. coli* cells. The donor plasmids (pFastBAC series) containing the foreign gene to be expressed are described in Chapter 3; essentially the gene of interest is inserted under the control of a baculovirus gene promoter, usually derived from the polyhedrin gene. *E. coli* cells (DH10Bac™, Invitrogen) that contain the baculovirus genome as a bacmid are then transformed with the donor plasmid and site-specific transposition generates the recombinant bacmid DNA. Transposition occurs between the mini-*att*Tn*7* target site in the bacmid DNA and a mini-Tn*7* element in the donor plasmid. Recombinant bacmid-containing colonies are obtained by plating onto selective media prior to amplifying a stock of *E. coli* cells. The recombinant bacmid DNA is then isolated from the *E. coli* cells using a simple alkaline lysis procedure (Subheading 3.8) and the resulting DNA is then used to transfect insect cells (Subheading 3.1). The culture medium harvested from the transfected cells contains recombinant virus only and requires no further selection prior to virus amplification (Subheading 3.4).

More recent advances have produced one-step baculovirus expression systems that involve the maintenance of a modified virus genome as a bacmid (in *E. coli* cells) and production of recombinant virus that requires no purification or selection steps (neither in bacterial cells nor insect cells) [9–12]. This technology has been commercialized as *flash*BAC™ (Oxford Expression Technologies) [13]. The *flash*BAC™ system utilizes a baculovirus (AcMNPV) genome that lacks part of an essential gene (ORF 1629) and contains a bacterial artificial chromosome (BAC) at the polyhedrin gene locus, thereby replacing the polyhedrin coding region. The essential gene deletion prevents virus replication within insect cells but the BAC allows the viral DNA to be maintained and propagated, as a circular genome, within bacterial cells [11, 12].

A recombinant baculovirus is produced by transfecting insect cells with the bacmid DNA and a transfer vector plasmid containing the foreign gene (Subheading 3.1). Homologous recombination within the insect cells restores the function of the essential gene allowing the virus DNA to replicate and produce virus particles. In addition, it simultaneously (1) inserts "the foreign gene" under the control of the polyhedrin gene promoter and (2) removes the BAC sequence. The recombinant virus genome, with the restored essential gene, replicates to produce budded virus that can be harvested from the culture medium of the transfected insect cells (and forms a seed stock of recombinant virus). As it is not possible for nonrecombinant virus to replicate there is no need for any selection system.

Recombinant baculoviruses can also be made using the BaculoDirect™ expression system (Life Technologies) [14]. In this system, the foreign gene is inserted directly from a Gateway® entry clone (described further in Chapter 3) into a linearized baculovirus genome that has been modified to contain *att*R sites. The gene of interest is simply cloned into a suitable Gateway entry vector and is then mixed with the BaculoDirect™ linear DNA and Gateway LR Clonase™. The resulting recircularized baculovirus DNA containing the foreign gene is then transfected into insect cells to generate recombinant virus through the use of standard protocols such as the one described under Subheading 3.1.

The following protocols cover the essential stages in making and selecting recombinant baculoviruses. Under Subheading 3.1 a generic protocol for the transfection of insect cells with virus DNA and transfer plasmid DNA (or bacmid DNA) is described. The end result of transfection will be a mixture of parental and recombinant virus if traditional baculovirus vector DNAs are used. The next step will be a plaque-assay to separate recombinant virus from nonrecombinants and this is described under Subheading 3.2. If the newer bacmid technologies are used, e.g., *flash*BAC™ or Bac-to-Bac®, then the transfection medium will only contain recombinant virus and no further selection step is required. Therefore, you can proceed directly to recombinant virus amplification as described under Subheading 3.4. The virus amplification method described under Subheading 3.4 is a general protocol that gives rise to high titer virus stocks; however, all virus stocks should be titrated by plaque-assay to determine an accurate titer (Subheading 3.5) or real time PCR (Subheading 3.6) [15] before proceeding to expression studies. Subheading 3.7 contains a generic method for the transformation of Bac-to-Bac DH10Bac™ cells with pFastBac donor plasmids and Subheading 3.8 provides a method for the isolation and purification of recombinant bacmid DNA that can then be used to transfect insect cells (Subheading 3.1). More details on these specific protocols can be found on the Life Technologies website [14].

## 2 Materials

### 2.1 Co-transfection of Insect Cells with Virus DNA and Transfer Vector DNA

1. Purified virus DNA that forms the basis of the recombinant virus expression vector (this may be purchased as part of a kit or as a stand-alone component). Use 100 ng [5 μL at 20 ng/μL] per co-transfection.
2. Transfer vector DNA containing the gene to be expressed in a designated transfer vector compatible with the baculovirus DNA used in **step 1**. Use 500 ng [5 μL at 100 ng/μL] per co-transfection (i.e., a fivefold excess over virus DNA). Any vector designed for double crossover, homologous recombination with baculovirus DNA at the polyhedrin locus is suitable. The DNA must be sterile and must be of a quality suitable for transfection into cells.
3. *Spodoptera frugiperda* (Sf-9 or Sf-21) insect cells. These are available commercially from suppliers such as Invitrogen and from the American Type Culture Collection (Rockville, USA). Alternatively, any laboratory working with the baculovirus expression system may be able to help. Further details on insect cell culture can be found in [16] and in Chapters 6, 7 and 9.
4. 35 mm tissue culture treated dishes seeded with insect cells (*see* **step 3**) in a sub-confluent monolayer.
5. Serum-free insect cell culture medium (*see* Chapter 8). If using serum-supplemented medium, then you will need medium with and without 10 % (v/v) fetal bovine serum.
6. Transfection reagent. Many different reagents are suitable for use in insect cells. The following are ones that we have used successfully, but it is by no means an exhaustive list: Lipofectin® (Life Technologies), FuGENE 6 (Promega), GeneJuice® (EMD Millipore), Tfx-20™ (Promega).
7. Incubator set at 27–28 °C.
8. 1 % Virkon (Amtec) or other suitable disinfectant.
9. Inverted phase-contrast microscope.
10. Plastic box to house dishes in the incubator.
11. Sterile pipettes, bijoux or similar vessels to prepare the transfection. Plasticware used to prepare the transfection mixture should be made from polystyrene and not from polypropylene.

### 2.2 Plaque-Assay to Separate Recombinant Virus from Parental Virus

1. 35 mm tissue culture treated dishes (about 14 dishes per transfection).
2. Insect cells (Sf-9 or Sf-21 cells) taken from a healthy, exponential growth phase culture (*see* Chapter 1 in this book regarding the exponential growth phase). The use of Sf-21

cells in serum-supplemented medium is strongly recommended for plaque-assays since they produce distinct, large plaques in 3 days, compared to smaller less distinct plaques in 4 days for Sf-9 or Sf-21 cells grown in serum-free medium.

3. Co-transfection medium to be titrated (Subheading 3.1).
4. Appropriate culture medium for the cells being used.
5. Low Gelling Temperature Agarose for cell culture (use 2 % w/v in sterile $dH_2O$, sterilized by autoclaving). Small aliquots of 15 mL are convenient and can be prepared in advance and stored solidified at room temperature. Melt in a microwave oven or boiling water bath just prior to use.
6. Antibiotics (optional) (penicillin and streptomycin prepared with 5 unit/mL penicillin G sodium and 5 μL/mL streptomycin sulphate in 0.85 % saline; 1:100 final dilution). Antibiotic use is optional but if used should be added to all media.
7. Incubator at 27–28 °C and a plastic sandwich box.
8. Phosphate-Buffered Saline (PBS, sterilized by autoclaving), pH 6.2.
9. Neutral Red (e.g., from Sigma). Prepare a stock solution at 5 mg/mL in water, filter through a 0.2 μm filter and store at room temperature. For use, dilute 1:20 in PBS (do not store after dilution).
10. 2 % (w/v) X-gal in dimethylformamide (DMF) to distinguish *lac*Z-positive plaques from *lac*-Z-negative plaques.
11. Sterile pipettes, tips, bijoux or similar containers to make serial dilutions.
12. Discard for virus waste, e.g., 1 % Virkon (Amtec) or other suitable disinfectant.
13. Inverted phase-contrast microscope.

### 2.3 Plaque-Purification of Recombinant Virus

1. Sterile Pasteur pipettes.
2. Appropriate cell culture medium dispensed in 0.5 mL aliquots.

### 2.4 Amplification of Recombinant Virus (Small-Scale)

1. Seed stock or plaque-pick of virus as inoculum.
2. 50–200 mL culture of exponential growth phase insect cells (Sf-21 or Sf-9) in appropriate medium (serum-free medium is best).
3. Shake culture flask (e.g., 1 L sterile glass or disposable Erlenmeyer flask) or spinner flask (e.g., 1 L Bellco Glass spinner flask). Note, flasks for monolayer culture of cells can be used for the purpose of virus amplification, but the virus titer achieved will generally not be as high as with cells in suspension culture.

4. Shaker (for shake flask cultures) or magnetic stirrer, etc.
5. Incubator set at 27–28 °C. (For spinner flask or tissue culture flask culture.)
6. Inverted phase-contrast microscope.
7. Sterile pipettes.
8. Disinfectant for discard.

### 2.5 Titration of Virus by Plaque-Assay

1. 35 mm tissue culture treated dishes (ten dishes per virus to be titrated).
2. Insect cells (Sf-9 or Sf-21 cells) taken from a healthy, exponential growth phase culture. The use of Sf-21 cells in serum-supplemented medium is strongly recommended for plaque-assays, as they produce distinct, large plaques in 3 days, compared to smaller less distinct plaques in 4 days for Sf-9 or Sf-21 cells grown in serum-free medium.
3. Amplified virus to be titrated (from Subheading 3.4).
4. Appropriate culture medium for the cells being used.
5. Low Gelling Temperature Agarose (e.g., Sigma Aldrich) for cell culture (use 2 % w/v in sterile $dH_2O$, sterilized by autoclaving). Small aliquots of 10 mL are convenient and can be prepared in advance and stored solidified at room temperature, which can be melted in a microwave oven just prior to use.
6. Antibiotics (optional) (penicillin and streptomycin prepared with 5 units/mL penicillin G sodium and 5 μL/mL streptomycin sulphate in 0.85 % saline; 1:100 final dilution). Antibiotic use is optional but if used should be added to all media.
7. Incubator at 27–28 °C and a plastic sandwich box.
8. Phosphate-Buffered Saline (PBS, sterilized by autoclaving), pH 6.2.
9. Neutral Red (e.g., from Sigma). Prepare a stock solution at 5 mg/mL in water, filter through a 0.2 μm filter and store at room temperature. For use, dilute 1:20 in PBS (do not store after dilution).
10. Sterile pipettes, tips, sterile containers to make serial dilutions.
11. Discard for virus waste, e.g., 1 % Virkon (Amtec) or other suitable disinfectant.
12. Inverted phase-contrast microscope.

### 2.6 Titration of Virus by Quantitative PCR

1. Recombinant budded virus stock(s) to be analyzed and an AcMNPV reference stock ($>1 \times 10^8$ pfu/mL, recently titrated [<1 month]). Virus stocks should be clarified via low speed centrifugation before titration.

2. High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals). Other kits could be used, but the protocol described below was optimized using the above kit so we strongly advise its use.
3. Quantitative PCR Mastermix, e.g., Absolute Blue QPCR ROX Mix (ABgene).
4. Optical 96-well plates and adhesive plate seal.
5. 96-well plate rotor for low speed centrifugation.
6. Ice box.
7. Sterile 1.5 and 0.2 mL microtubes (optional 0.5 mL).
8. Sterile pipette tips.
9. Real-Time PCR system, such as Applied Biosystems 7500 Real-Time PCR Sequence Detection System.
10. Forward primer 2.5 μm Gp64-F109089* (5′ CGGCGTGA GTATGATTCTCAAA).

    Reverse primer 2.5 μm Gp64-R109026* (5′ ATGAGCAGAC ACGCAGCTTTT).

    Probe 2.5 μm Gp64-P109075* (5′ AAAAGTCTACGTTCAC CACGCGCCAAA).

    TaqMan probe labelled with FAM (6-carboxy-fluorescein) at the 5′ end and a quencher, TAMRA (6-carboxy-tetramethylrodamine) at the 3′ end. *Relative to position within the AcMNPV genome [17].

### 2.7 Transformation of E. coli DH10Bac™ with pFastBac Vectors to Produce Recombinant Bacmid (Bac-to-Bac® System)

1. Purified pFastBac vector containing gene to be expressed (200 pg/μL in TE, pH 8).
2. *E. coli* DH10Bac™ cells ready for transformation (supplied by Life Technologies as part of a kit or separately) [14].
3. Selective Luria broth (LB) agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal and 40 μg/mL IPTG. Three freshly prepared plates are required for each transformation. One liter LB is prepared by mixing 10 g tryptone, 5 g yeast extract and 10 g sodium chloride in 1 L water and adjusting the pH to 7.0. Sterilize by autoclaving. LB-agar is prepared by adding 15 g/L agar before autoclaving. After autoclaving, cool to "hand-hot", add antibiotics and other selective agents, and pour into standard 90 cm bacterial plates.
4. LB for growth of *E. coli* cells (see above).
5. 15 mL disposable, sterile centrifuge tubes.
6. 42 °C water bath.
7. 37 °C shaking and non-shaking incubator.

### 2.8 Isolation of Recombinant Bacmid DNA for Transfection into Insect Cells (Bac-to-Bac® System)

1. LB medium containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline (Subheading 2.6).
2. Solution A: 15 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 μg/mL RnaseA. Sterilize by filtration and store at 4 °C.
3. Solution B: 0.2 M NaOH, 1 % w/v SDS. Filter sterilize and store at ambient temperature.
4. 3 M potassium acetate, pH 5.5. Sterilize by autoclaving and store at 4 °C.
5. Isopropanol and 70 % v/v ethanol.
6. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.

## 3 Methods

### 3.1 Co-transfection of Insect Cells with Virus DNA and Transfer Vector DNA

1. For each co-transfection to make a recombinant virus, you will require one 35 mm dish of insect cells (Sf-9 or Sf-21) (*see* **Note 1**). It is also advisable to set up one dish as a mock-transfected control.
2. Seed the dishes with insect cells at least 1 h before use. It is extremely important to use healthy exponential growth phase cultures and to seed the cells at the correct cell density so that the resulting monolayer is even and sub-confluent. As a guide, use $1.0–1.5 \times 10^6$ cells/dish in a 2 mL volume of medium.
3. Ensure that the cells are evenly distributed over the surface of the dish and leave to settle at room temperature for 1 h on a flat surface.
4. During the 1 h incubation period in **step 2**, prepare the co-transfection mix of DNA and liposome reagent. For each co-transfection, pipette 1 mL serum-free, antibiotic-free medium into a sterile, disposable polystyrene container (7 mL bijoux are convenient).
5. Add an appropriate volume of transfection reagent as directed by the manufacturer and mix. As a guide, use 5 μL Lipofectin® (or alternative transfection reagent).
6. Add 100 ng virus DNA and 500 ng transfer vector. Mix with gentle agitation. Omit the DNA from the medium in the mock-transfection control.
7. Incubate at room temperature for 15–30 min to allow the liposome-DNA complexes to form.
8. Just before the end of the incubation period in **step 7**, remove the culture medium from the 35 mm dishes of cells using a sterile pipette; ensure that the cell monolayer is not disrupted.
9. If using cells maintained in serum-supplemented medium, then wash the monolayer twice with serum-free medium before

carrying out the co-transfection. This is to remove any residual serum, which inhibits liposome-mediated transfection of DNA into cells. Carefully add 1 mL serum-free medium then remove and discard medium. Repeat once. When removing liquid from a dish of cells, tip the dish at a 30°–60° angle so the liquid pools towards one side the dish. This washing step is not necessary when using cells maintained in serum-free medium

10. As soon as the medium has been removed from the cells, add the 1 mL of DNA + liposome complex dropwise and gently to the center of each dish; so as not to disturb the monolayer. Incubate in a plastic sandwich box at 27–28 °C for a minimum of 5 h or overnight.
11. After the incubation period add a further 1 mL of the appropriate insect cell culture medium to each dish. If the cells are normally maintained in serum-supplemented medium, then add 1 mL of medium containing 10 % serum at this step.
12. Continue the incubation for 3–5 days in total, according to the recommendations given with the source of baculovirus vector DNA. For example, with the *flash*BAC system (Oxford Expression Technologies) 5 days are strongly recommended (*see* **Note 2**).
13. Following the incubation period, harvest the medium containing the recombinant virus into a sterile container, and store in the dark at 4 °C until used (*see* **Notes 3–5**).
14. With the exception of the *flash*BAC expression system, the harvested transfection medium will contain a mixture of recombinant and nonrecombinant virus. The next step will be to separate recombinant virus from nonrecombinant virus, as described under Subheading 3.2. With the *flash*BAC system, only recombinant virus can replicate after transfection and the next step will be to amplify a stock of recombinant virus (Subheading 3.4).

### 3.2 Plaque Assay to Separate Recombinant Virus from Parental Virus

1. To separate recombinant virus plaques from nonrecombinant virus plaques, prepare about 12 dishes of cells per transfection experiment (Subheading 3.1). Seed the dishes with an appropriate number of cells to form a sub-confluent monolayer (normally $1.4 \times 10^6$ Sf-21 cells or $0.9 \times 10^6$ Sf-9 cells/dish) (*see* **Note 6**). Place the dishes on a level surface at room temperature for 1 h to provide time for the cells to recover.
2. During this incubation period (**step 1**) prepare serial log (1 in 10) dilutions of the transfection medium. Prepare dilutions from $10^{-1}$ to $10^{-3}$. It is convenient to prepare 0.5 mL dilutions by placing 0.45 mL of the appropriate medium into each of three sterile bijoux (or micro centrifuge tubes).
3. Add 50 μL of undiluted transfection medium into the first bijou (this will be $10^{-1}$) and mix thoroughly by vortex or inversion.

Using a fresh pipette tip, remove 50 μL from this bijou and transfer it to the next one (this will be $10^{-2}$) and vortex/mix. Continue diluting the virus in this way to the required dilution. You will also need 0.5 mL medium for the control dishes.

4. About 1 h after seeding the dishes (**step 1**), check that the cells have formed an even, sub-confluent monolayer. When ready to add the virus dilutions to the cells, remove the culture medium and discard into 1 % Virkon or other disinfectant. Ensure that the cell monolayer is not disrupted during this process and that it does not dry out. It is best to leave a small amount of medium to just cover the cells—if the monolayer dries out this will give rise to a large shiny pink area, devoid of live cells, after staining the plaque assay.
5. Add 100 μL from each of the dilutions from $10^{-1}$ to $10^{-3}$ to triplicate dishes of cells and add 100 μL undiluted transfection mixture to three other dishes. Add the diluted virus dropwise to the center of each dish, using a fresh sterile pipette for each, and label accordingly. Also include two dishes as controls where 100 μL of the appropriate insect cell culture medium is added to each dish, in place of a virus dilution.
6. Incubate the dishes at room temperature for 1 h on a level surface for virus adsorption. Do not leave the dishes for longer than 1 h (40 min is the minimum). It is important to ensure that the cell monolayer does not dry out at this stage. If working in a Class II biological safety cabinet, then remove the dishes to the bench at this stage to prevent the cells from drying out.
7. About 15 min prior to the end of the virus adsorption period, prepare the LGT agarose overlay. Completely melt a 15 mL aliquot of ready-prepared and solidified 2 % (w/v) LGT agarose (in a microwave oven or boiling water bath, taking appropriate safety precautions) and, after cooling to about 50 °C (hand-hot), add an equal volume (15 mL) of appropriate insect cell culture medium. Mix thoroughly but gently, avoiding air bubbles. You need 2 mL of this agarose/medium overlay for each dish. Use immediately or keep warm at 45 °C to prevent solidification. If using a water bath, then ensure that it is clean and wipe the bottle of overlay with alcohol before use to prevent fungal/bacterial contamination of the plaque-assay. If using antibiotics, then add to the culture medium before preparing the overlay. Should the agarose set before using, do not remelt it; prepare a fresh batch.
8. After the virus adsorption period and after preparing the agarose overlay, carefully remove the virus inoculum from each of the 35 mm dishes by tipping the dish to one side and using a sterile Pasteur pipette. Discard into 1 % Virkon or other disinfectant.

Take care not to disturb the cell monolayer or allow it to dry out during this process.

9. Gently pipette 2 mL of the agarose-overlay down the side of each dish, allowing it to roll over the cells, so as not to disturb the monolayer. Incubate at room temperature for 15 min or until the agarose is solid. The time taken for the agarose to solidify depends on the room temperature.
10. When the agarose overlay has set, add 1 mL of appropriate insect cell culture medium to each dish as a liquid feed overlay. Antibiotics may be added to the medium if desired.
11. Place the dishes into a secure container (e.g., a sandwich box) and incubate at 28 °C for 3 days (Sf-21 cells) or 3–4 days (Sf-9 cells), by which time the cell monolayer should be confluent (with no gaps between cells).
12. Once the cells have reached confluence, the dishes can be stained with Neutral Red and/or X-gal in order to visualize the plaques. Plaques are clear areas against a red background as only live cells take up the Neutral Red stain. *Lac*Z-positive virus plaques (e.g., nonrecombinant or parental virus plaques) can be stained using X-gal and Neutral Red.
13. Remove the liquid overlay from the dishes and replace with 1 mL diluted Neutral Red stain (and 15 μL 2 % w/v X-gal) and incubate for at least 5 h (may need overnight) at 27–28 °C. Plaques will be colorless in a background of red cells. *Lac*-Z-positive plaques will appear blue when X-gal has been added.
14. Tip off the stain (into disinfectant) and invert dishes (place on tissue paper which can then be discarded after autoclaving). Replace lids. Leave the dishes in the dark, in the inverted position, for the plaques to clear. This may take a few hours or may occur very rapidly, depending on the strength of the Neutral Red.
15. After staining, plaques should be visible on at least one of the various dilutions plated. If plaque-purification is required (where the transfection mixture yields a mixture of the two), then the next step will be to pick plaques and amplify them (Subheading 3.3). Where the transfection yields only recombinant virus, you can proceed to the amplification of a stock virus (Subheading 3.4).

### 3.3 Plaque-Purification of Recombinant Virus

1. Select well-isolated plaques (colorless recombinant plaques, if using blue-white selection), preferably from a plate in which there are no contaminating parental virus (blue) plaques. If this is not possible, then pick a well-isolated, clear plaque.
2. Using a sterile Pasteur pipette (preferably), take up a plug of agarose from the center of a plaque. Disperse the plug into 0.5 mL appropriate culture medium.

3. Disperse the virus in the plug by vortexing.
4. If needed, use this virus as a stock to perform another plaque-assay (Subheading 3.2) for a further round of plaque-purification.
5. Plaque-purification should continue until a plaque can be picked from a plate in which there are no contaminating parental virus plaques.
6. The 0.5 mL plaque-pick virus can be used to amplify a stock of recombinant virus (Subheading 3.4).

### 3.4 Amplification of Recombinant Virus

1. Prepare a 50–200 mL culture of Sf-9 or Sf-21 cells at an appropriate cell density (use exponential growth phase cells (*see* **Notes 7–9** and Chapter 1)). If preparing cells in monolayer flasks, then seed so that they form a sub-confluent monolayer.
2. Using aseptic technique, add 0.5 mL (no more) (*see* **Note 7**) of the recombinant virus seed stock to the cell culture and incubate with shaking or stirring (as appropriate) until the cells are well infected (normally 4–5 days) (*see* **Note 9**). In monolayer flasks (75 $cm^2$), remove the medium and add approximately 0.1 mL virus and 0.4 mL medium to each flask. Ensure that this is distributed evenly over the surface of the cell monolayer for 1 h. Remove the virus-containing medium and replace with an appropriate volume of fresh medium (10–15 mL in a 75 $cm^2$ tissue culture flask).
3. When the cells appear well infected with virus, harvest the culture medium and remove cells by low speed centrifugation, at 4 °C for 15 min. Decant aseptically and store the recombinant virus in the dark at 4 °C. The virus inoculum may be stored for 6–12 months or longer in the dark at 4 °C. The titer of the virus will start to fall after storage for more than 3–4 months and therefore it is recommended that the virus be re-titered prior to use (e.g., *see* Subheadings 2.5 and 3.5). Note that re-amplification may be required. Many laboratories have observed that the titer decreases much more rapidly in serum-free medium and that the addition of 2–10 % serum significantly stabilizes the virus during long-term storage at 4 °C (*therefore, the addition of serum is highly recommended!*). Virus may also be frozen at −80 °C for a longer period of time but avoid multiple freeze and thaw cycles. Upon freezing, the virus titer may decrease and should be re-amplified and re-titered when thawed. Do not store virus at −20 °C or in liquid nitrogen.
4. It is strongly recommended that an accurate titer be obtained prior to using the virus in experiments (e.g., *see* Subheadings 2.5 and 3.5) (*see* **Notes 10–14**).

### 3.5 Plaque Assay to Titrate Amplified Virus

Alternative methods of titering the virus are given in Chapters 5, 10, 11, and 22 in this book.

1. To titrate an amplified virus by plaque assay, seed ten dishes of cells per virus. Seed the dishes with an appropriate number of cells to form a sub-confluent monolayer (normally $1.4 \times 10^6$ Sf-21 cells or $0.9 \times 10^6$ Sf-9 cells/dish). Leave the dishes for 1 h on a level surface at room temperature for the cells to recover. Sf-21 cells are preferred for plaque-assays as they give more distinct, larger plaques in a shorter period of time (*see* **Note 6**). The cells must be healthy and taken from exponential growth phase.
2. During this incubation period prepare serial log (1 in 10) dilutions of the virus to be titrated, i.e., dilutions from $10^{-1}$ to $10^{-7}$. It is convenient to prepare 0.5 mL dilutions by placing 0.45 mL appropriate medium into each of seven sterile bijoux (or micro centrifuge tubes).
3. Add 50 μL of undiluted recombinant virus from the amplified stock of virus or transfection mix to the first bijoux (this will be $10^{-1}$) and mix thoroughly by gentle vortexing or inversion. Using a fresh pipette tip, remove 50 μL from these bijoux and transfer it to the next one (this will be $10^{-2}$) and vortex/mix. Continue diluting the virus in this way to the required dilution. You will also need 0.5 mL medium for the control dishes.
4. About 1 h after seeding the dishes (**step 1**), confirm that the cells have formed an even, sub-confluent monolayer. Just prior to adding the virus dilutions to the cells, remove the culture medium and discard into 1 % Virkon or other disinfectant. Ensure that the cell monolayer is not disrupted during this process and that the cells do not dry out. It is best to leave a small amount of medium to just cover the cells—if the monolayer dries out this will give rise to a large shiny pink area, devoid of live cells, after staining the plaque assay.
5. Add 100 μL from each of the dilutions from $10^{-4}$ to $10^{-7}$ to duplicate dishes. Add the diluted virus dropwise to the center of each dish, using a fresh sterile pipette for each, and label accordingly. Also include two dishes as controls where 100 μL of the appropriate insect cell culture medium is added to each dish, in place of a virus dilution.
6. Incubate the dishes at room temperature for 1 h on a level surface for virus adsorption. Do not leave the dishes for longer than 1 h (40 min is the minimum). It is important to ensure that the cell monolayer does not dry out at this stage. If working in a Class II biological safety cabinet, then remove the dishes to the bench at this stage to prevent the cells from drying out.

7. About 15 min prior to the end of the virus adsorption period, prepare the LGT agarose overlay. Completely melt a 10 mL aliquot of ready-prepared and solidified 2 % (w/v) LGT agarose (in a microwave oven or boiling water bath, taking appropriate safety precautions) and, after cooling to about 50 °C (hand-hot), add an equal volume (10 mL) of the appropriate insect cell culture medium. Mix thoroughly, but gently, avoiding air bubbles. 2 mL of this agarose/medium overlay is used for each dish. Use immediately or keep warm at 45 °C to prevent solidification. If using a water bath, then ensure that it is clean and wipe the bottle of overlay with alcohol before use to prevent fungal/bacterial contamination of the plaque-assay. If using antibiotics, then add to the culture medium before preparing the overlay. Should the agarose set before using, then a fresh batch should be prepared, i.e., do not re-melt it.
8. After the virus adsorption period and after preparing the agarose overlay, carefully remove the virus inoculum from each of the 35 mm dishes by tipping the dish to one side and using a sterile Pasteur pipette. Discard into 1 % Virkon or other disinfectant. Take care not to disturb the cell monolayer or allow it to dry out during this process.
9. Gently pipette 2 mL of the agarose-overlay down the side of each dish, allowing it to roll over the cells, so as not to disturb the monolayer. Incubate at room temperature for 15 min or until the agarose is solid. The time taken for the agarose to solidify depends on the temperature of the room.
10. When the agarose overlay has set, add 1 mL of appropriate insect cell culture medium to each dish, as a liquid feed overlay. Antibiotics may be added to the medium if desired (Subheading 2.2).
11. Place the dishes into a secure container (e.g., a sandwich box) and incubate at 28 °C for 3 days (Sf-21 cells) or 4 days (Sf-9 cells), by which time the cell monolayer should be confluent (with no gaps between cells).
12. Once the cells have reached confluence, the dishes can be stained with Neutral Red and/or X-gal in order to visualize the plaques. Plaques are clear areas against a red background as only live cells take up the Neutral Red stain. *Lac*Z-positive virus plaques can be stained using X-gal and Neutral Red (Subheading 3.2).
13. Remove the liquid overlay from the dishes and replace with 1 mL diluted Neutral Red stain and incubate for at least 5 h (may need overnight) at 27–28 °C. Plaques will be colorless in a background of red cells. Lac-Z-positive plaques will appear blue when X-gal has been added.

14. Tip off the stain (into disinfectant) and invert dishes (place on tissue paper which can then be discarded by autoclaving). Replace lids. Leave the dishes in the dark in the inverted position for the plaques to clear. This may take a few hours or may occur very rapidly, depending on the strength of the Neutral Red.
15. After staining, determine the titer of a virus by selecting one set of duplicate dishes with between 10 and 30 plaques (ideally) and count them. Calculate the average number of plaques for that dilution and calculate the virus titer (*see* **Notes 15** and **16**).

### 3.6 Titration of Virus by Quantitative PCR

1. Following the manufacturer's instructions, isolate viral DNA from 200 μL of recombinant budded virus using the viral nucleic acid isolation kit. Elute the DNA in elution buffer to a final volume of 50 μL. Store at 4 °C until required.
2. Prepare a log series of tenfold dilutions of the AcMNPV standard in final volumes of 200 μL to $10^3$ pfu/mL and extract DNA as described in **step 1**. Once established, the standard curve may be reused for future QPCR analyses.
3. Prepare QPCR reactions on ice as a master mix depending upon how many virus samples you need to titrate (*see* Table 1). Multiply the amounts shown in Table 1 (1 QPCR column) by the number of reactions required. Control reactions contain water instead of DNA. The probe is light sensitive and therefore should be stored in the dark as much as possible.
4. Aliquot 23 μL of master mix from **step 3** into the appropriate number of wells in a 96-well plate. Each reaction should be carried out in triplicate, i.e., 3 × 5 wells for standards, 3 wells for water control and 3 wells per unknown virus.
5. To each set of triplicate wells add 2 μL of the purified virus DNA from **step 1** or 2 μL of the DNA standards (**step 2**) or control water, to give a final reaction volume of 25 μL in each well.

**Table 1**
**Components for QPCR master mix reaction**

| Reagents | 1 QPCR (μL) | Example<br>1 set of AcMNPV DNA standards + control samples + 5 virus DNA samples + 1 extra reaction[a] (μL) |
|---|---|---|
| PCR SuperMix-UDG | 12.5 | 437.5 |
| Water | 7.5 | 262.5 |
| Forward primer (2.5 μM) | 1 | 35 |
| Reverse primer (2.5 μM) | 1 | 35 |
| Probe (2.5 μM) | 1 | 35 |
| | 23 | 805 |

[a]Ensures that there is sufficient volume to allow for pipetting errors

6. Seal the 96-well plate using an adhesive plate seal and plastic spreader.
7. Centrifuge the 96-well plate briefly at low speed to bring the reactions to the bottom of the wells. Ensure there are no bubbles on the surface of the reactions.
8. Place the 96-well plate within the Sequence Detection System (SDS) and enter the required information into the software, e.g., the position of each reactions, the fluorescent dyes used (6FAM and TAMRA) and the standard DNA dilutions.
9. Perform the DNA amplification following the manufacturer's instruction using the cycling conditions detailed in Table 2.
10. On completion of the QPCR cycle program, the most exponential part of each of the amplification curves will have been automatically detected by the SDS software and their Ct values calculated from the default threshold (*see* Fig. 1). Occasionally, this may need to be adjusted manually and the threshold moved higher in the exponential phase of amplification to give improved slope and correlation coefficient values. However, where possible, the baseline setting and threshold

**Table 2**
**SDS cycling conditions**

| Stage 1: UNG activation | Stage 2: Taq activation & UNG deactivation | Stage 3: Annealing/ extension |
|---|---|---|
| Cycles: 1<br>50 °C<br>2:00 | Cycles: 1<br>95 °C<br>15:00 | Cycles: 40<br>95 °C/60 °C<br>0:15/1:00 |

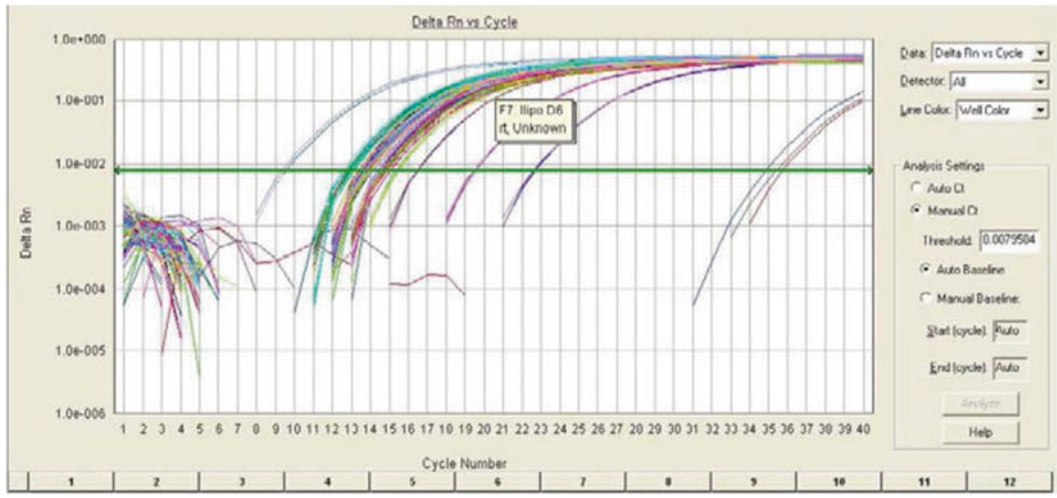


**Fig. 1** Typical amplification plot from an Applied Biosystems SDS 7500

levels should remain consistent between assays to improve accuracy and reproducibility. Ideally, the standard curve should have an R2 value >0.95 and an amplification efficiency of around −3.2.

11. Export the Ct values into a data analysis program (e.g., Microsoft Excel) and calculate the mean Ct for each virus. Construct a standard curve for the AcMNPV dilutions and use it to determine titers of the unknown samples.

### 3.7 Transformation of E. coli DH10Bac™ with pFastBac Vectors to Produce Recombinant Bacmid (Bac-to-Bac® System)

1. Take one tube of competent *E. coli* DH10Bac™ cells (Invitrogen) and thaw on ice.
2. For each transformation, add 100 μL thawed cells into a 15 mL centrifuge tube (prechilled on ice). Then add 1 ng (5 μL) pFastBac plasmid construct containing the gene to be expressed. Mix very gently by tapping the tube.
3. Incubate the cells and DNA on ice for 30 min.
4. Transfer the tube to a water bath at 42 °C for 2 min (heat shock).
5. Transfer the tube into ice and chill for 2 min.
6. Add 900 μL Luria broth medium (or similar) and shake cells at 37 °C for 4 h to allow expression of the antibiotic resistance markers.
7. Prepare tenfold serial dilutions of the cells ($10^{-1}$–$10^{-3}$) with LB and plate out 100 μL aliquots of each dilution onto selective agar plates (Subheading 2.7). Plate several of each dilution.
8. Incubate the plates at 37 °C for at least 48 h to allow the blue color to develop in colonies arising from nonrecombinant bacmids. Colonies arising from cells containing recombinant bacmids will be large and white. Avoid selecting any colonies that are grey or have a slightly dark center.
9. Well-isolated, large white colonies should be selected and used to isolate bacmid DNA ready for transfection into insect cells (Subheading 3.1).
10. It is advisable to check the purity of the 'white' colonies selected by re-streaking onto selective agar plate (Subheading 2.7) prior to amplifying and purifying bacmid DNA. Select 5–10 colonies and streak onto selective agar plates using standard microbiological techniques. Incubate for 24–48 h until the bacterial growth is clear and well established. Select isolated colonies to amplify cells for bacmid DNA isolation (Subheading 3.8).

### 3.8 Isolation of Recombinant Bacmid DNA for Transfection into Insect Cells (Bac-to-Bac® System)

1. Using a sterile bacterial loop, select a single, isolated colony from the streak plate (Subheading 3.7) and use to inoculate 2 mL LB medium containing antibiotics (Subheading 2.7). Grow culture overnight at 37 °C in a shaking incubator. The following is a standard alkaline lysis procedure for isolating plasmid DNA.

2. Transfer 1.5 mL of culture to a microcentrifuge tube and pellet cells at 14,000 × *g* for 1 min.
3. Remove culture medium and resuspend cell pellet in 0.3 mL ice-cold solution A.
4. Add 0.3 mL solution B and mix gently. Incubate at ambient temperature for 5 min until the solution clears.
5. Slowly add 0.3 mL 3 M potassium acetate, mix and place on ice for 5–10 min. A precipitate should form.
6. Remove the precipitate by centrifugation at 14,000 × *g* for 5–10 min and then transfer the lysate to a new tube. Add 0.8–1.0 mL isopropanol, mix by inversion and place on ice for 10–15 min.
7. Pellet the DNA by centrifugation at 14,000 × *g* for 10–15 min and wash the DNA pellet with 70 % ethanol.
8. Remove the supernatant, air dry the DNA pellet and then resuspend in 40–50 μL sterile TE. Store at 4 °C (not frozen). Do not shear the DNA by pipetting too often. Keep sterile since this DNA will be transformed into insect cells.
9. Before using the recombinant bacmid DNA to transfect insect cells and produce recombinant virus (Subheading 3.1), it is recommended that the DNA is analysed by PCR or other methods to confirm recombinant bacmid integrity.

## 4 Notes

1. Do not use *Trichoplusia ni* cells for the production or amplification of recombinant virus. Use these cells only for protein production.
2. Cell monolayers in which recombinant virus has been produced will appear very different from mock-transfected control cells under the inverted microscope. Control cells will have formed a confluent monolayer, whereas virus-infected cells will not have formed a confluent monolayer and will appear grainy with enlarged nuclei.
3. If the instructions above have been followed and the insect cells are in good condition, then the recombinant virus titer produced after co-transfection will normally be high (extensive testing in our labs indicate an average titer of about $10^7$ pfu/mL at 5 days).
4. The cells remaining from the co-transfection may be used to test for foreign gene expression, e.g., by Western blot analysis.
5. In our experience, the recombinant virus titer produced during the co-transfection is not adversely affected by using semi-pure transfer plasmid DNA (e.g., that produced by resin-based

mini-prep DNA protocols); however, the foreign gene expression levels in these initial infected cells are significantly higher if good quality transfer vector DNA is used. Subsequent levels of expression using the recombinant virus to infect fresh cell cultures are not affected by the quality of transfer vector DNA.

6. Sf-21 cells are preferred for plaque-assays as they give more distinct, larger plaques in a shorter period of time. The cells must be from healthy exponential phase cultures.
7. The cell density that should be used will vary with the cell type and the method of culture. As a guide use Sf-9/Sf-21 cells in shake culture, in serum-free medium, at $2 \times 10^6$ cells/mL or Sf-21/Sf-9 cells, in serum-supplemented medium, in spinner culture at $0.5 \times 10^6$ cells/mL. It is important that the cells are healthy and in exponential growth phase to ensure that virus replication occurs efficiently to amplify high virus titer stocks for subsequent use in expression studies. You should check the cell density and viability before using the cells to amplify virus. It is also critically important that the cells are infected at a very low multiplicity of infection (MOI) (<1 pfu/cell), thereby resulting in an initial infection of only a fraction of the cells within the culture. The fraction of cells infected at a given MOI can be estimated by using the Poisson distribution (*see* Chapter 1). The cells that are infected will then produce BV that will infect remaining uninfected cells (i.e., a secondary infection). Following this methodology will significantly reduce the probability of defective interfering particle accumulation (*see* Chapter 1). In contrast, if the cells are infected at high MOI (≫1 pfu/cell), then all the cells will be infected by the primary infection (i.e., only one round of replication will occur) and greatly enhance the probability of defective interfering particle accumulation.
8. Do not use *T. ni* cells for this purpose (*see* **Notes 1–5**).
9. Virus-infected cells become uniformly rounded and enlarged, with distinct enlarged nuclei. They appear grainy when compared with healthy cells under the phase-contrast inverted microscope. The oxygen demand of the cells increases following virus infection and therefore it becomes increasingly important that the surface area to volume ratio be as large as possible for maximum gas exchange—do not overfill flasks!
10. If it is desired to simultaneously infect all of the cells within the culture in order to have a synchronous culture, then it is critical that the cells be infected with a known and relatively high MOI (an MOI of 10–20 is commonly used for this purpose).
11. Initial infection of all of the cells within the culture (*see* **Note 10**) also maximizes the chance of detecting the expressed protein—especially where the levels of expression are at the lower end of the scale! It also minimizes the chances of protein degradation

becoming a problem (e.g., protease degradation of secreted proteins).

12. Sometimes, for unknown reasons, virus amplifications do not work (although the reason is normally that the cells were not healthy or were not in the exponential growth phase, or that the cells were infected at too high an MOI; *see* **Note 7**). Thus, it is important to use healthy cells from the exponential growth phase and to use only virus that has been properly amplified and titered in order to avoid the disappointment resulting from very low or undetectable gene expression.
13. The most common cause of failure to detect foreign gene expression is using a virus stock in which the titer is assumed to be high, but is actually low.
14. For most purposes a titer of $5 \times 10^7$ pfu/mL or higher is adequate. A titer of $<10^7$ pfu/mL will not normally be sufficient for expression studies.
15. Calculation of virus titer from raw data:
    Titer of virus (pfu/mL) = average plaque count × dilution factor* × 10**

    *multiply by the inverse of the dilution used on the plate used to count the plaques

    **multiply by 10 because only 0.1 mL was applied to each dish.

    Example: 25 plaques (average) on the $10^{-6}$ dilution plates give a titer of:

    $$25 \times 10^6 \times 10 = 25 \times 10^7 = 2.5 \times 10^8 \text{ pfu/mL}$$
16. If virus does not amplify well, then there are normally two main reasons. First, the cells were not in good condition or were not used from the exponential growth phase. Second, too much virus was added in the inoculum, thereby resulting in an initial infection of the all the cells in culture (*see* **Note 7**).

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# Chapter 5

# Gene Expression in Mammalian Cells Using BacMam, a Modified Baculovirus System

**James A. Fornwald, Quinn Lu, Frederick M. Boyce, and Robert S. Ames**

## Abstract

BacMams are modified baculoviruses that contain mammalian expression cassettes for gene delivery and expression in mammalian cells. BacMams have become an integral part of the recombinant mammalian gene expression toolbox in research labs worldwide. Construction of transfer vectors is straightforward using basic molecular biology protocols. Virus generation is based on common methods used with the baculovirus insect cell expression system. BacMam transduction of mammalian cells requires minimal modifications to familiar cell culture methods. This chapter highlights the BacMam transfer vector pHTBV.

**Key words** BacMam, Modified baculovirus, Mammalian expression, Transient expression, Viral transduction, Co-expression, Pseudotyping, Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)

## 1 Introduction

The BacMam gene delivery and expression method has been an integral tool supporting drug discovery research at GlaxoSmithKline (GSK) for more than a decade. While relatively simple and quick to produce, BacMam viruses have significantly reduced the need for tedious stable mammalian cell line generation that has previously been a workhorse experimental process for decades. BacMam transduction has also significantly reduced our use of other transient transfection methods for mammalian gene expression. Although virus generation requires an additional 2 weeks for reagent preparation in comparison to mammalian expression plasmids, the benefits of applying BacMams are evident in the many articles and reviews [1–4] published since the original discovery was described [5, 6]. BacMams have proven to be an efficient and inexpensive way to express recombinant mammalian genes both for large scale short-term drug discovery screening efforts and for future reproducible follow-up experiments.

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BacMams derived from the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) [7] transduce a variety of mammalian cell lines, including HEK-293, HeLa, U-2 OS, monkey kidney (COS), and Chinese Hamster ovary (CHO) cells [8, 9]. The viral genome can stably accommodate an insert sequence of at least 38 kb [10], thereby making expression of large and multiple genes possible. BacMams have been used as a delivery vehicle to mammalian cells for many gene classes, including nuclear receptors [11], secreted proteins [12–14], and membrane proteins, e.g., G protein-coupled receptors (GPCRs) [15–17], drug transporters [18], and ion channels [19]. Applications range from functional characterization and protein production for structural studies [20], to cell membrane generation [21], high content screening assay development [22], and vaccine production [23]. With its high transduction efficiency and flexibility, the technology easily enables co-expression applications and modulation of expression level by timing of exposure to and dosing of BacMams (Fig. 1).

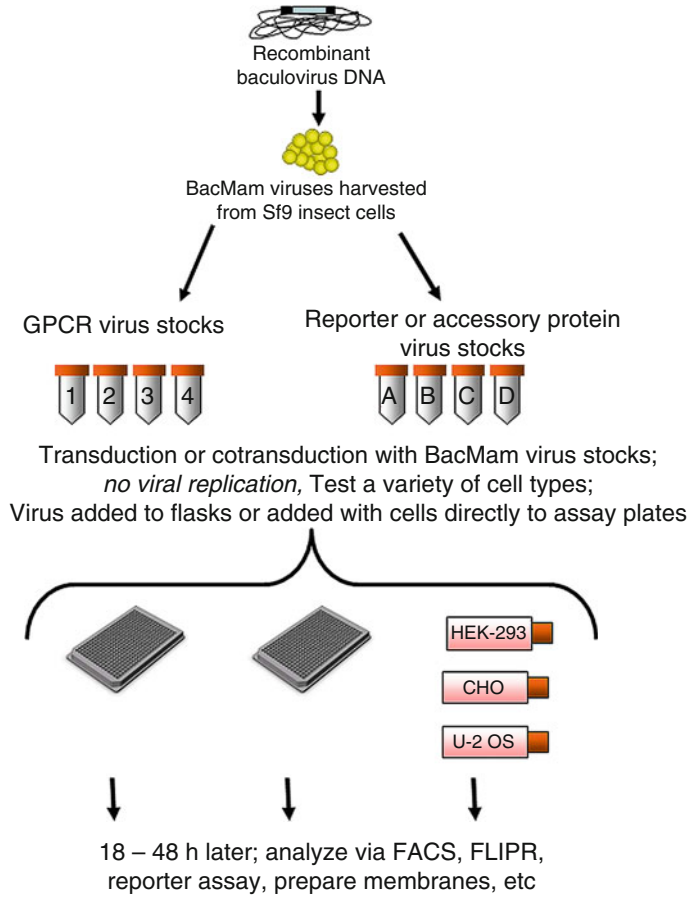


**Fig. 1** Schematic representation of BacMam generation and subsequent transduction of mammalian cells for functional studies and assay development

An important element of routine drug discovery screening assays is the requirement for reproducible batches of reagents through the entire process from assay development, high throughput screening, to any downstream follow-up experiments. We routinely use frozen mammalian host cells [24] to add another level of reproducibility in the transduction process. U-2 OS, HEK-293, and CHO cells all perform well from frozen cell stocks and together they form our core set of BacMam hosts. U-2 OS cells are ideal for HTS assays—they are firmly adherent, highly transducible, and have a curiously low background of endogenously expressed GPCRs [15]. HEK-293 cells also transduce well but are less adherent. We engineered HEK-293 cells to express the macrophage scavenger receptor (MSR) to enhance adherence to plastic assay plate wells. The GripTite™ 293 MSR Cell Line is commercially available from Invitrogen. CHO cells are less efficiently transduced and significant effort has gone into improving expression of human cytomegalovirus IE1 (hCMV-IE1) promoter driven constructs in CHO cells [13, 25, 26].

Since the 1980s the baculovirus expression system has been in widespread use for high level protein expression in insect cells. Improvements to virus generation systems have significantly reduced the time and the level of expertise required to overexpress gene products in insect cells. The two common virus generation systems are the Bac-to-Bac™ system available from Invitrogen and the BD BaculoGold™ system from BD Biosciences. Both systems use a relatively small transfer vector to facilitate construct generation. With the Bac-to-Bac™ method, recombination of transfer vector elements into the baculoviral genome takes place through a transposition event in an engineered DH10bac strain of *E. coli*; with the BaculoGold™ system, the recombinant baculoviral genome is generated in insect cells via homologous recombination. The Bac-to-Bac™ system is our workhorse system and virus generation is predictable for most constructs. In our laboratory we use the BaculoGold™ system as a back-up when needed.

Given that the virus does not replicate in mammalian cells it is essential to maximize both internalization of the virus particle and transcriptional efficiency of the expression cassette. To support new assay formats in a larger set of host cells we have added the optimized pHTBV vectors. These second generation vectors incorporate several enhancements (*see* Subheading 3.1.4) for improved host range and gene expression (*see* Subheading 3.7.1). The vectors are engineered to incorporate the Vesicular Stomatitis Virus G protein (VSV-G) in the virus coat during production in the insect cell host. In addition, the mammalian expression cassette includes an optimized hCMV-IE1 promoter with an intron in the 5′ untranslated region (UTR) and the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) in the 3′ UTR.

While we do not have a full understanding of the mechanism of BacMam virus transduction of mammalian cells, studies have shown that altering the virus coat can be beneficial [27, 28]. Wild type baculoviruses have evolved to efficiently infect insect cells using the baculoviral coat protein gp64. Although transduction efficiency varies significantly among different mammalian cell lines, experiments have shown that similar amounts of virus are internalized despite differing transduction efficiencies, suggesting that internalization is not the determining factor in mammalian cell transduction [6]. VSV-G pseudotyping of BacMam viruses has improved mammalian cell transduction efficiency, perhaps by allowing more efficient escape from endosomes [27], without significantly affecting virus production in the insect cell host (unpublished observations). Over-expression of the baculovirus viral coat protein gp64 has also been reported to enhance transduction [28].

Transcript splicing plays a role in enhancing mRNA levels and translation efficiency [29], but many common mammalian expression vectors do not include any intron, or they include it in the suboptimal 3′ UTR location. Some intronless mammalian genes are known, but compensating mechanisms are believed to play a role in maintaining required expression levels. Numerous reports indicate that cDNAs deleted of introns are less efficiently expressed than the same gene with the intact wild type genomic structure. Gene sequences often extend over large stretches of DNA so the only practical solution is to use the cDNA for recombinant expression systems. Insertion of introns proximal to the promoter is a compromise solution that can often be beneficial [30].

The pHTBV vectors include the WPRE element in the 3′ UTR. The WPRE has been shown to post transcriptionally enhance retroviral gene expression [31] and is commonly used in lentivirus vectors. A tenfold improvement in BacMam transduction was reported in some cell lines when a WPRE was introduced into the 3′ UTR [32]. One advantage of BacMam over other viral mammalian expression systems is the ability to transduce nondividing cells. A BacMam constructed with a WPRE was used to transduce embryonic stem-cell derived neurons with transgene expression detectable for 3 months [33].

BacMams enable unprecedented facile assay development involving multiple components. Correct functional recombinant expression of mammalian genes often occurs only within the environment of the mammalian cell and co-expression of partner proteins may be required. The partner proteins may be endogenously expressed in the host, but we frequently transduce host cells with two or more BacMams simultaneously for over-expression of protein complexes or signal transduction pathways (Fig. 1).

## 2 Materials

1. Baculovirus transfer vector pFastbac1 (Invitrogen, Carlsbad, CA).
2. An expression cassette from a mammalian vector, e.g., pcDNA3.1 (Invitrogen).
3. pHTBV1.1 (Rick Boyce, Mass. General Hospital).
4. Restriction endonucleases.
5. Plasmid purification kit (Qiagen, Waltham, MA).
6. Sf-9 cells (ATCC or Invitrogen).
7. A serum-free insect cell medium: HyClone SFX (Thermo Scientific, Logan, UT) or Ex-Cell 420 (Sigma-Aldrich, St. Louis, MO).
8. Heat-inactivated fetal bovine serum (FBS, Invitrogen).
9. Kanamycin, tetracycline, IPTG.
10. Gentamicin and Bluo-gal (Invitrogen).
11. Luria Broth (LB) ampicillin plus gentamicin plates (with 70 μg/mL ampicillin and 2 μg/mL gentamicin).
12. *E. coli* strain DH10Bac (Invitrogen).
13. S.O.C. recovery medium (Invitrogen).
14. DH10Bac plates: Luria Agar plates plus, 50 μg/mL kanamycin, 2 μg/mL gentamicin, 5 μg/mL tetracycline, 40 μg/mL IPTG, 100 μg/mL Bluo-gal.
15. DH10Bac growth medium: LB medium supplemented with 50 μg/mL kanamycin, 2 μg/mL gentamicin, 5 μg/mL tetracycline.
16. Plasmid solutions are as follows. P1 is 50 mM Tris–HCl, pH 8.0, 10 mM EDTA. P2 is 200 mM NaOH, 1 % SDS. P3 is 3.0 M potassium acetate, pH 5.5 (Qiagen Buffer Set).
17. Transfection reagent Cellfectin (Invitrogen) or FuGENE HD (Promega, Madison, WI).
18. Centrifuge for cell culture, Allegra 6, GH-3.8A rotor (Beckman Coulter, Brea, CA).
19. 500-mL and 3-L Shake flasks for Sf-9 cell cultures (Corning, Acton, MA).
20. Cell culture flasks T-25, T-75, and T-150 (Corning).
21. Sterile Falcon polypropylene culture tubes, 5, 15, and 50 mL (Becton Dickinson, Franklin Lakes, NJ).
22. The BacPAK Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA).
23. DMEM/F12 medium for mammalian cell cultures (Invitrogen).
24. Trypsin (SAFC Biosciences, St. Louis, MS).

25. Dulbecco's phosphate buffered saline (DPBS) with 0.1 mM EDTA.
26. Versene (Invitrogen).
27. BacMam GFP transduction control (Invitrogen, catalog #B10383).
28. Histone deacetylase inhibitors, e.g., sodium butyrate or trichostatin A.
29. Optional filter units for clearing virus stocks (Nalgene MF75 0.2 μm, Thermo Scientific).

## 3 Methods

### 3.1 BacMam Transfer Vector Construction

We routinely use the Bac-to-Bac™ baculovirus expression system (Invitrogen) for BacMam virus generation. The Bac-to-Bac™ system is based on the method of Luckow et al. [7], which uses the Tn7-mediated site-specific transposition reaction to direct the integration of the transfer vector expression cassettes into a baculovirus backbone vector (bacmid) in the *E. coli* strain DH10 Bac. The bacmid is a mini-F replicon with the baculovirus genome and a kanamycin resistance marker. In addition, DH10Bac contains a helper plasmid containing a Tn7 transposase gene and a tetracycline resistance marker. The system was designed in such a way that the recombinant Tn7 transposon from the transfer vector will be integrated into a mini-*att*Tn7 site in the *lac*Zα gene contained within the recombinant viral genome, causing inactivation of the α-complementation of lacZ. The desired recombinant transformants will be resistant to tetracycline, kanamycin, and gentamicin and can be easily distinguished from nonrecombinants by blue/white selection on X-gal plates.

The system relies on a small and easily engineered transfer vector for construction of the expression cassette. There are few commercially available transfer vectors with basic BacMam functions so we built a set of vectors. Construction of BacMam transfer vector backbones for use with this virus generation system starts with removal of the strong viral polyhedrin promoter from pFastBac1 and introduction of the mammalian expression cassette. Following subcloning of the cDNA downstream of the hCMV-IE1 promoter (or other mammalian promoter), the recombinant baculoviral genome is constructed by transforming the transfer vector into the DH10Bac strain (*see* Subheading 3.2.1). The recombinant viral bacmid DNA is isolated and used to transfect insect cells to generate the recombinant virus. The entire process is simple and easy to perform, allowing generation of multiple viruses simultaneously. With this procedure, recombinant BacMam virus stocks can be generated in 2 weeks.

The initial BacMam transfer vectors described by Boyce [6] were based on the BaculoGold™ virus generation method. With the introduction of Bac-to-Bac™ system, a BacMam transfer vector based on the pFastbac1 virus generation method and the pcDNA3 mammalian expression cassette (pFastBacMam1) was described [9].

The BacMam transfer vectors described here are derivatives of pFastBac1 of the Bac-to-Bac™ system (Invitrogen). In these vectors, the insect expression cassette in pFastBac1 is replaced by a mammalian expression cassette. All other features of the Bac-to-Bac™ system are retained. Thus, the procedure to generate a BacMam is identical to that used to generate a recombinant baculovirus for insect cell expression.

#### 3.1.1 *pFastBac1*

pFastBac1 (Fig. 2a) is a standard transfer vector commercially available from Invitrogen for baculovirus generation. It contains the strong viral polyhedrin promoter, a multiple cloning site (MCS), and the SV40 polyA sequence. The Tn7 transposition cassette also contains a gentamicin resistance marker to facilitate selection of the recombinant bacmid in DH10Bac cells. This vector is used as a backbone vector to accommodate DNA inserts containing mammalian expression cassettes (i.e., the target gene flanked by a mammalian promoter and a poly A sequence). For generation of our in-house vectors we replaced the polyhedrin promoter with a mammalian expression cassette from existing mammalian expression vectors.

#### 3.1.2 *pFastNot1*

A useful vector backbone is pFastNot1 (Fig. 2b) which can be used for delivering any DNA sequence into cells susceptible to transduction. Cloning a mammalian expression cassette into the pFastbac1 backbone produces the basic BacMam which is suitable for transduction of receptive host cells for short-term expression experiments. Following construction of the BacMam transfer vector, virus generation follows the same procedure used to make any baculovirus. pFastNot1 is a derivative of pFastBac1 in that the polyhedrin promoter in pFastBac1 is removed. This was done by deleting DNA sequences contained between SnaBI and StuI sites in pFastBac1. The vector is smaller than pFastBac1, and is used to accommodate any DNA fragments intended for introduction into mammalian cells, including mammalian expression cassettes.

#### 3.1.3 *pFastBacMam-1*

pFastBacMam-1 (Fig. 2c) has been described previously [9]. The vector is a hybrid of pFastBac1 and pcDNA3 (Invitrogen), which contains the mammalian expression cassette with the hCMV-IE1 promoter, a MCS, and the BGH polyadenylation sequence. In addition, it contains a neomycin resistance marker for selection of stable cell lines following BacMam transduction if desired [9].

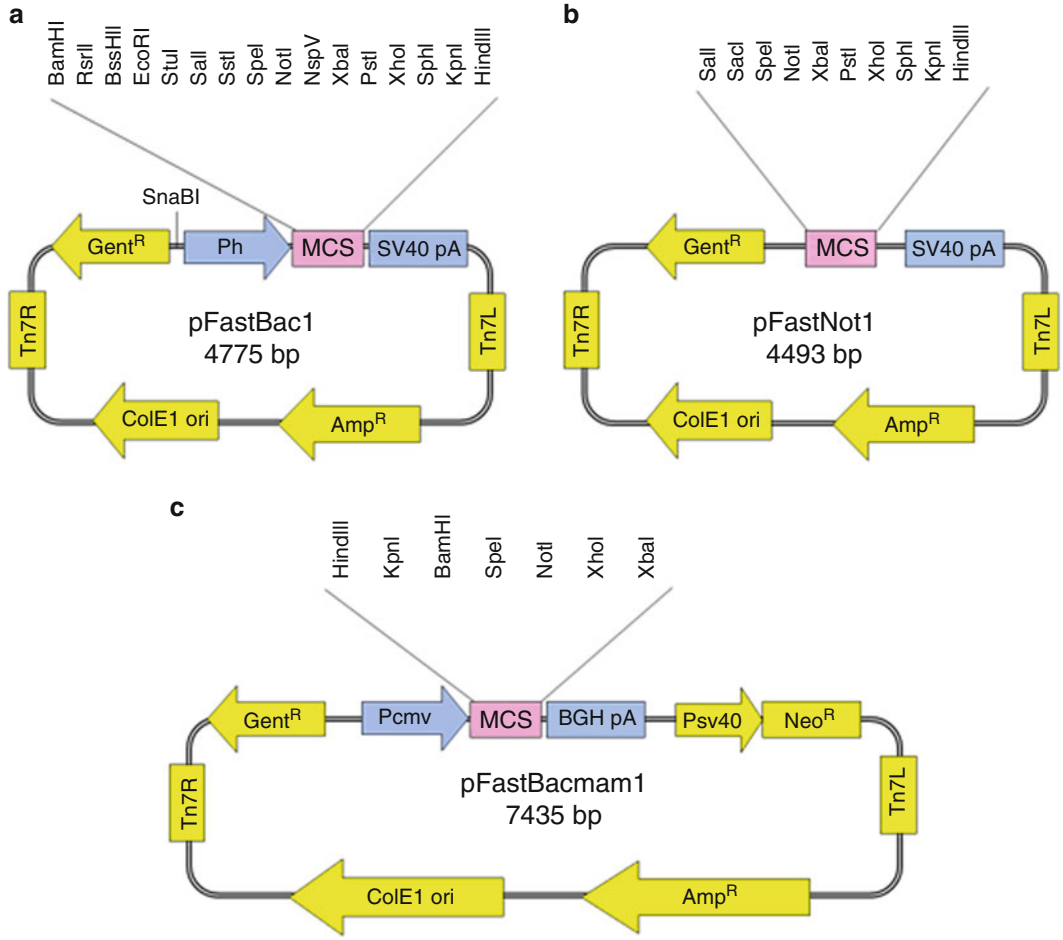


**Fig. 2** First generation BacMam transfer vectors constructed in-house using the pFastbac1 backbone. (**a**) pFastBac1 (Invitrogen). (**b**) pFastNot1 (*see* Subheading 3.1.2). (**c**) pFastBacmam1 (*see* Subheading 3.1.3). Gent$^R$, gentamicin resistance gene; Ppolh, polyhedrin promoter; MCS, multiple cloning site; SV40 pA, SV40 polyadenylation signal; Tn7R, Tn7L, *right* and *left* arms of the Tn7 transposon; Amp$^R$, ampicillin resistance gene; ColE1 ori, ColE1 origin of replication; Pcmv, human cytomegalovirus promoter; BGH pA, bovine growth hormone polyadenylation signal; Psv40, SV40 early promoter and origin; Neo$^R$, Neomycin resistance gene

*3.1.4 pHTBV*

The construction of the pHTBV vectors represents a significant improvement in the aspect of enhanced host transduction and efficient expression of the recombinant gene. The new features incorporated into the vector include the VSV-G (GenBank ACK77583, amino acids 1-511), the WPRE (GenBank J04514, base pairs 1093-1684), and the herpes virus CMV-IE1 promoter containing the 5′ UTR-intron (GenBank M60321, base pairs 561-1317). The pHTBV vector is the best expressing BacMam vector that we have tested.

The vector can be used as a Gateway destination vector if your cDNA vectors include Gateway recombination sequences (Fig. 3a).

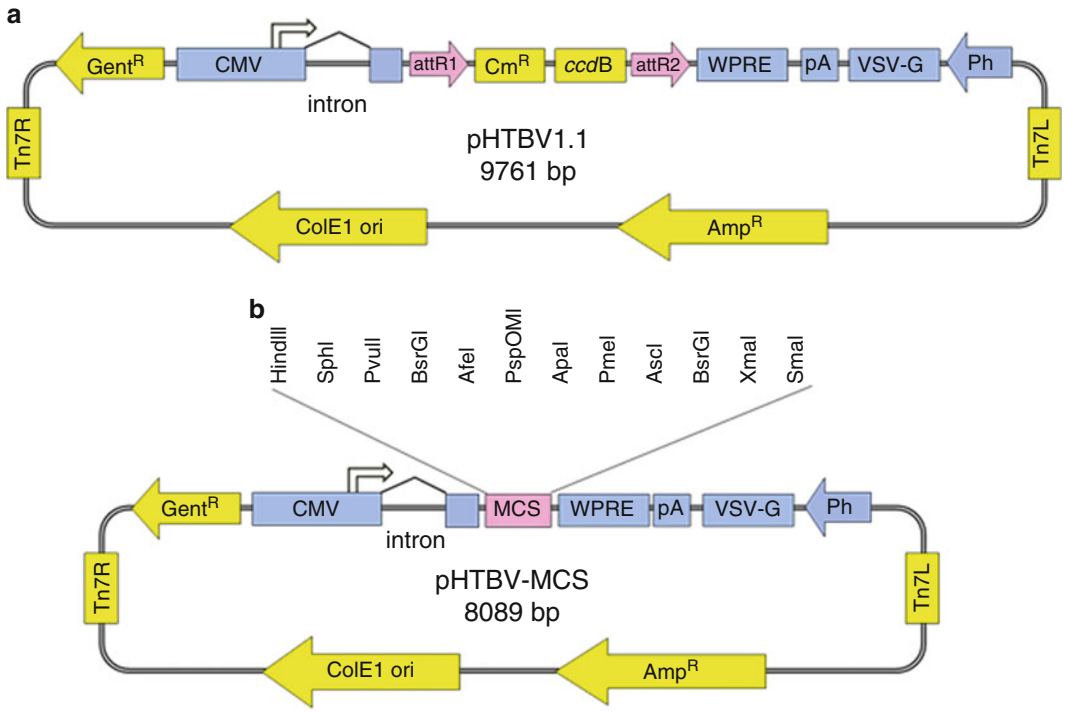


**Fig. 3** Second generation pHTBV enhanced BacMam transfer vectors for Gateway (**a**) or restriction enzyme cloning (**b**). *Bent arrow*, human cytomegalovirus promoter transcription start site; intron, partial intron A, human cytomegalovirus IE1; attR1, attR2, Gateway DNA recombination sites; $Cm^R$, chloramphenicol resistance gene; *ccd*B, cytotoxic *ccd*B gene; WPRE, Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element; pA, SV40 polyadenylation signal; Ph, polyhedrin promoter; VSV-G, Vesicular Stomatitis Virus G gene

We use the Gateway system for subcloning cDNAs when they are available as compatible entry clones, but detailed protocols for Gateway cloning system (Invitrogen) are beyond the scope of this chapter. The principal advantage of using this system is that the cDNA is more easily subcloned into the small entry vector than into the larger BacMam vector.

We have also deleted the Gateway sequences (between *att*R1 and *att*R2) and introduced a region with multiple unique cloning sites for subcloning cDNAs by restriction enzyme cloning (Fig. 3b).

1. Digest pHTBV 1.1 (from Rick Boyce) with HindIII and partial digest with BsrGI.
2. Oligonucleotides were synthesized to introduce restriction enzyme cloning sites in the digested pHTBV1.1. To construct pHTBV-MCS (Fig. 3b) the following oligos were annealed and ligated into the vector prepared in **step 1**:

   5′AGCTTGCATGCCAGCTGTACAGCGCTGGGCCCGTT
   TAAACTGGCGCGCCTGTACACCCGG

   and

5′GTACCCGGGTGTACAGGCGCGCCAGTTTAAACGGG
CCCAGCGCTGTACAGCTGGCATGCA

The pHTBV vector with Gateway sequences deleted (Fig. 3b) is over 8000 bp so the number of available unique restriction sites is limited.

#### 3.1.5 cDNAs

Cloned cDNAs of genes of interest are obtained from many sources and carried on a variety of plasmid vectors. If a significant number of genes are subcloned from a common vector, then it is useful to configure a BacMam transfer vector with compatible restriction sites. Gateway recombination (Invitrogen) and InFusion (Clontech) cloning are common approaches for construct generation. Detailed protocols are available from the kit suppliers.

#### 3.1.6 Subcloning Using Restriction Enzymes

Subcloning of a cDNA ORF into a BacMam transfer vector is carried out using standard recombinant DNA procedures.

1. Isolate the gene insert from its vector by restriction digestion or PCR.
2. Ligate the gene insert with appropriately digested transfer vector DNA.
3. Transform *E. coli* (Top10, DH5α, etc.) for identification of recombinants.
4. Plate transformants on LB ampicillin plus gentamicin plates (*see* **Note 1**).
5. Screen recombinant clones by restriction analysis of miniprep DNA or analytical PCR.
6. Desired clones are further confirmed by DNA sequencing across the insert region.

### 3.2 Generation of Bacmid DNA

The recombinant BacMam bacmid DNA is isolated using the same protocol used for generating a baculovirus for insect cell expression as discussed in Subheadings 3.2.1 and 3.2.2.

#### 3.2.1 Transformation of DH10Bac

1. Transform 15–20 μL of DH10Bac competent cells (Invitrogen) with 10 ng BacMam transfer plasmid in a 15 mL culture tube.
2. Incubate on ice for 30 min.
3. Heat shock 42 °C for 30 s.
4. Add 1 mL S.O.C. recovery medium.
5. Incubate at 30 °C for 3–4 h.
6. Pellet the culture, decant the supernatant, and resuspend the cells in the residual broth which remains in the culture tube (~150 μL). Plate varying amounts of culture on three DH10Bac plates (e.g., 10, 50, 90 μL).
7. Incubate at 30 °C for 2 days.

8. Pick three large white recombinant lacZ-negative colonies; streak each for single colonies on a DH10Bac plate (*see* **Note 2**).
9. Incubate at 30 °C for 2 days.

#### 3.2.2 Preparation of Bacmid DNA

Bacmid DNA is isolated using a modified protocol of the alkaline lysis method [35]. The buffers (P1, P2, P3) included in the Qiagen DNA Maxi Preparation Kit are used, but solutions prepared from laboratory chemical stocks work as well. It is neither necessary nor desirable to do a column purification step to clean up the DNA, which often results in lower yields of the large bacmids.

1. Examine the plates of restreaked colonies generated under Subheading 3.2.1.
2. Identify two plates with large white colonies; pick one large white colony from each plate.
3. Inoculate each into 2 mL of DH10Bac growth medium.
4. Incubate overnight in a shaker at 30 °C (*see* **Note 3**).
5. Transfer 1.4 mL of the overnight culture to a 1.5-mL microfuge tube; pellet the cells at maximum speed for 1 min.
6. Resuspend the pellet in 200 μL P1; add 200 μL P2; invert to mix, incubate for 5 min at room temperature.
7. Add 200 μL P3; invert to mix; spin in a microcentrifuge at maximum speed for 10 min to pellet debris.
8. Transfer clarified supernatant to a sterile 1.5 mL microfuge tube. Add 420 μL isopropanol and invert to mix.
9. Spin in a microcentrifuge at maximum speed for 10 min to pellet the DNA. In a biosafety cabinet, remove the supernatant and wash the pellet with 70 % ethanol. Allow the DNA to dry and resuspend the bacmid DNA in 50 μL sterile $dH_2O$.

### 3.3 Generation of BacMam Virus

Perform all insect cell culture and virus handling procedures in a biosafety cabinet to maintain the sterility of the BacMam stocks for subsequent applications.

#### 3.3.1 Sf-9 Cell Culture

Sf-9 cells are excellent hosts for producing BacMam virus. Sf-9 suspension cultures are easy to establish (*see* **Note 4**) and grow very well as single cells in suspension. The doubling time of Sf-9 cells is 18–24 h and Sf-9 cultures are easily scaled to large volumes as needed. Cells adapted to HyClone SFX perform well for many passages (*see* **Note 5**) before a replacement culture is needed.

#### 3.3.2 Transfection of Sf-9 Cells

1. Exponentially growing cells are desired for transfection. Approximately $2.5 \times 10^6$ cells in 3.5 mL of HyClone SFX are seeded into a T-25 cell culture flask (*see* **Note 6**). The cells are allowed to attach for 15–30 min.

2. For each transfection, add 25 μL of bacmid DNA (from Subheading 3.2.2) to a 5-mL Falcon tube.
3. In a 50-mL Falcon conical tube, prepare enough transfection mix for all the bacmids: dilute 7 μL Cellfectin (Invitrogen) in 200 μL HyClone SFX medium for each sample. FuGENE HD (Promega) can be substituted (7 μL in 200 μL medium).
4. Add 200 μL of the transfection mix to the tube containing the bacmid DNA, mix briefly, and incubate at room temperature for 15 min.
5. Hold the T-25 flask (from **step 1**) at an angle to expose most of the cells, drip the DNA/lipid mix directly onto the cells.
6. The transfection flask is incubated overnight at 27 °C (*see* **Note 7**).
7. Add 3.5 mL of HyClone SFX medium supplemented with 10 % heat inactivated fetal bovine serum (FBS) to the flask.
8. Cover the flasks with a sheet of aluminum foil to reduce light exposure and continue the incubation for another 4 days at 27 °C or until lysis of the cells is evident.

### 3.3.3 Harvesting the P0 Virus

Cytopathic effect is usually evident 3–4 days after bacmid transfection. Areas of clearing (inhibition of cell growth, cell detachment, and lysis of cells) should be visible when the T-flask is held to a light. The pHTBV viruses express the fusogenic VSV-G protein during growth in Sf-9 insect cells so the infected monolayer shows evidence of larger cell clumps.

1. Transfer the conditioned culture medium from the T-flask to a conical 15-mL culture tube.
2. Centrifuge to pellet the cell debris for 10 min at 900×*g* (2000 rpm in a Beckman GH-3.8 rotor).
3. Transfer the supernatant containing the virus to a sterile 15-mL screw cap tube.
4. Store the virus at 4 °C in the dark.

Although P0 stocks are usually not titrated at this stage, the titers usually vary between stocks, typically in the range of $10^7$ plaque forming units per milliliter (pfu/mL).

### 3.3.4 Generating P1 Virus

Virus stocks are amplified further in Sf-9 suspension cultures. P1 stocks are routinely produced in 150-mL cultures in 500-mL Corning flasks.

1. Seed culture to $10^6$ cells/mL; incubate 27 °C overnight on a shaker at 120 rpm.
2. Cultures typically grow to 2.5–3×$10^6$ cells/mL, count samples from a few flasks.
3. Add heat-inactivated FBS (Invitrogen) to a final concentration of 5 % (*see* **Note 8**).

4. Infect with 0.2–0.5 mL P0 and incubate on a shaker (120 rpm) at 27 °C for 4 days in the dark (*see* **Note 9**).
5. Count the cells after 2 days to confirm infection (cell diameters increase, cell division slows).
6. Transfer the culture to a 250-mL conical centrifuge bottle; pellet debris at 4500 × *g* (4000 rpm in a Beckman JS-4.2 rotor) at 4 °C for 20 min.
7. The conditioned medium (P1 stock) is stored at 4 °C in the dark. P1 BacMam stock typically reaches a titer of $>5 \times 10^8$ pfu/mL, and following clarification by low speed centrifugation can be used to transduce mammalian cells.

#### 3.3.5 Large-Scale Amplification of BacMams

For larger volumes of virus stocks, one or more liter cultures are infected.

1. Transfer up to 1 L of HyClone SFX medium to a 3-L Corning shake flask for optimal culture aeration.
2. Seed Sf-9 cells to $10^6$ cells/mL, incubate culture on a shaker (85 rpm) at 27 °C in the dark.
3. After 24 h or when the cell counts reach $2.5–3 \times 10^6$ cells/mL, 0.5 mL P1 stock is added (*see* **Note 10**).
4. Add heat-inactivated FBS to 5 % (*see* **Note 8**).
5. After 4 days, transfer the culture to 500-mL centrifuge bottles.
6. Pellet cell debris at 4500 × *g* (4000 rpm in a Beckman JS-4.2 rotor) at 4 °C for 20 min.
7. Decant virus into sterile bottles or filter if desired.
8. Store at 4 °C in the dark. This P2 BacMam stock typically reaches a titer $>5 \times 10^8$ pfu/mL.

### 3.4 BacMam Virus Titration

Virus titration can be time consuming, but should be part of the standard quality control process. Any standard method for baculovirus titration can be used (e.g., *see* Chapters 4, 10, 11 and 22). Currently we routinely use the BaculoTiter Assay Kit (Invitrogen), but the BaculoTiter Sf-21 cells are no longer commercially available. The kit is useful for high throughput (96 or 384 well plate) determination of virus titers in 2 days.

Another method we have used routinely is based on the BacPAK Baculovirus Rapid Titer Kit (Clontech) which uses an anti-gp64 antibody to detect viral plaques on Sf-9 cell lawn. It also takes 2 days to perform although it is more labor intensive. The kit includes a complete protocol manual, but a brief outline of the protocol is included here for comparison.

1. Seed a 96-well plate with Sf-9 cells.
2. Infect individual wells with dilutions of virus.

3. Incubate 1 h; remove virus inoculum; overlay with methyl cellulose to impede spread of virus.
4. Incubate 2 days at 27 °C in the dark.
5. Stain plaques with mouse anti-gp64, goat anti-mouse antibody/HRP conjugate, and blue peroxidase substrate.
6. Blue plaques are visualized by microscopy and counted to determine the virus titer.

### 3.5 Long-Term Storage of BacMam Virus

BacMam virus stocks (P1 or P2) are stable for longer than 1 year when kept refrigerated and in the dark (*see* **Note 8**). Titers of P0 stocks will drop over time but can be used for many years for the generation of new P1 stocks. We routinely store BacMam viruses in liquid form at 4 °C. BacMam virus stocks can also be stored frozen but there is some loss of titer. Viruses can be stored as BIICs (baculovirus infected insect cells) [36] in liquid nitrogen.

### 3.6 Virus Purification

Procedures for recombinant baculovirus purification have been described [37]. However, for BacMam transduction into cultured cells, it is not necessary to purify the virus, i.e., the P1 and P2 stocks can be used directly (*see* **Note 11**).

### 3.7 Application of BacMams

BacMam transduction of mammalian cells is performed in the same medium and at the same incubation temperature (37 °C, 5 % $CO_2$) that is used to propagate the mammalian host cells (*see* **Note 12**).

#### 3.7.1 Identification of Receptive Mammalian Cell Lines Using a BacMam GFP Transduction Control

BacMam viruses will transduce a variety of mammalian cell lines (*see* **Note 13**). However, transduction efficiency varies among different cell lines. The BacMam transduction efficiency for any cell line can be determined by using a BacMam expressing green fluorescent protein. It is also useful for optimizing transduction conditions, such as culture medium, temperature, time, etc. The conditions used to transduce HEK-293 cells with an in-house GFP BacMam are noted in the legend of Fig. 4. The FACS can be used to generate quantitative data following transduction, but a fluorescent microscope or other imaging instrument can be used when available.

A quick way to determine if your cell line of interest is receptive to BacMam transduction is to use the commercially available pHTBV BacMam GFP Transduction Control (Invitrogen). We often refer to pHTBV derived viruses as "second generation" BacMams; Invitrogen refers to pHTBV as "BacMam 2.0" technology. Enhanced transduction of some cell lines is often evident for pHTBV derived BacMams compared to the "first generation" pFastBacMam1 derived BacMams (*see* Fig. 5). A BacMam GFP transduction control is useful for validating the transfer vector design, for example, comparing different mammalian promoters in the cell line of interest.

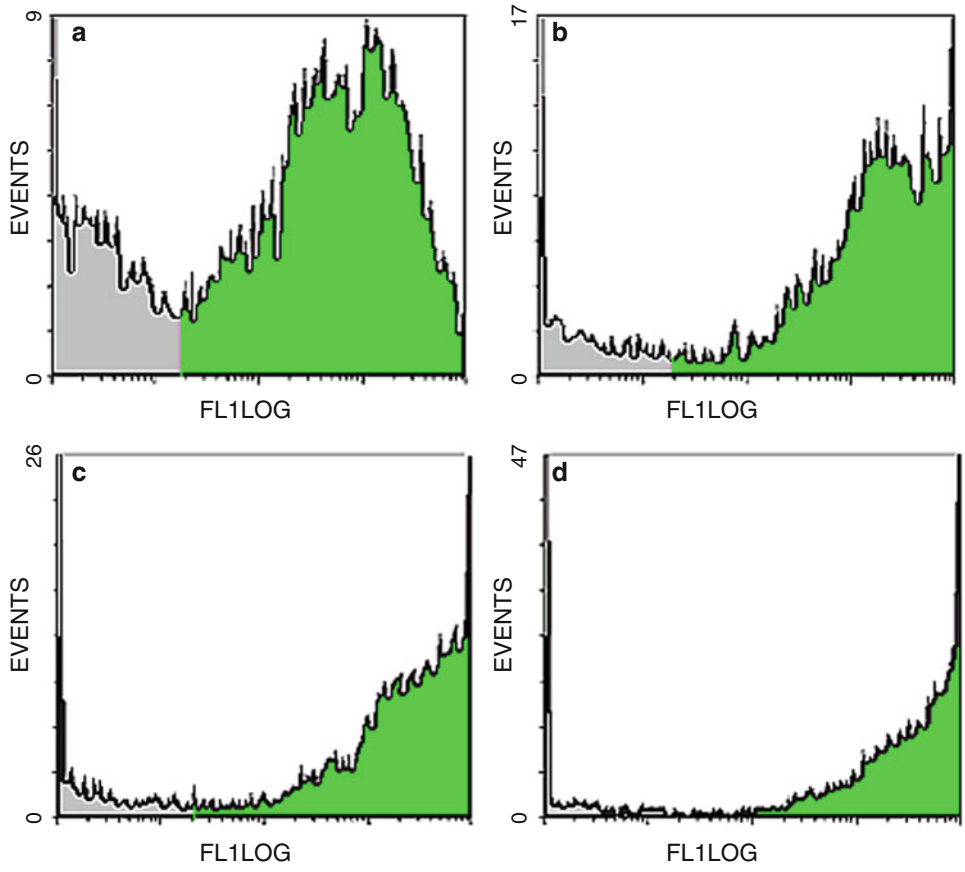


**Fig. 4** FACS analysis of HEK 293 cells transduced with BacMam expressing GFP. HEK 293 cells were transduced in a T-75 flask with varying amounts of first generation BacMam virus expressing GFP. (**a**) 0.05 mL (MOI ~ 3); (**b**) 0.25 mL (MOI ~ 15); (**c**) 0.5 mL (MOI ~ 30); (**d**) 1 mL (MOI ~ 60)

*3.7.2 Transduction of Adherent Mammalian Cells*

Adherent cells (e.g., HEK-293, CHO-K1, U-2 OS) are maintained in T-150 flasks in DMEM/F12 + 10 % FBS (complete medium) and passaged every 4–5 days at split ratios between 1:10 and 1:20. Cells harvested from subconfluent flasks are often used to achieve optimal transduction efficiency. BacMam transduced cells prepared by this procedure are used in place of stable cell lines. They can be stained with antibodies, used for membrane preparations or in functional assays (*see* **Note 14**). If larger numbers of cells are required (e.g., for protein purification), then suspension cultures are often preferred.

1. Aspirate the growth medium. Wash the cell layer with 10 mL Dulbecco's phosphate buffered saline (DPBS) with 0.1 mM EDTA. Add 3–5 mL of 0.05 % trypsin and incubate briefly at room temperature. Dislodge the cells and transfer cells to a 50-mL tube containing 15 mL complete medium. Pellet the cells; gently resuspend in 10 mL complete medium.

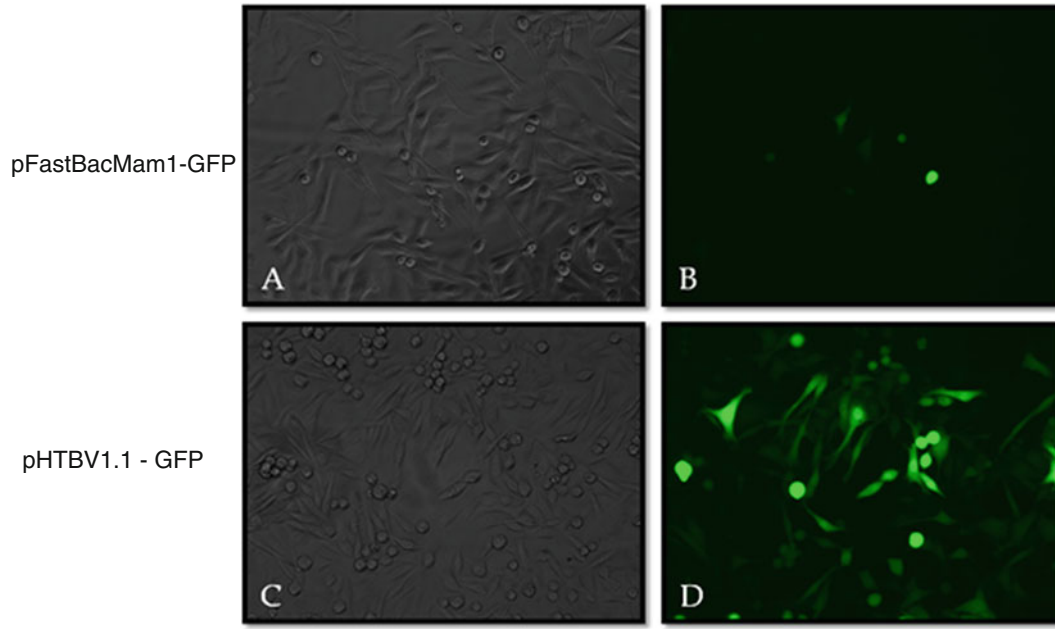


**Fig. 5** Comparison of relative transduction efficiency of the first generation pFastBacMam1-GFP and second generation pHTBV-GFP viruses in A-204 cells. Cells were seeded into a 96 well plate at $10^4$ cells per well in RPMI 1640 (Invitrogen 11875) + 10 % FBS. Virus was added ($5 \times 10^6$ pfu per well) and images (magnification 200×) were captured 48 h post-transduction. Images were captured using white light (**a**, **c**) or UV light (**b**, **d**)

2. Count the cells and plate $1.5 \times 10^6$ cells in 30 mL complete medium in a T-75 flask.
3. Add varying amounts of virus to flask. MOIs of 5–100 are routinely tested. For a stock with no titer information, one can generally assume that the titer is $>10^8$ pfu/mL. However, it is strongly recommended that accurate titers be determined (even though it can briefly delay initiation of transduction experiments) to have confidence that a low viral titer is not the source of expression problems.
4. Incubate the flask overnight at 37 °C with 5 % $CO_2$.
5. Aspirate the medium, wash with 5 mL DPBS/0.1 mM EDTA (*see* **Note 15**).
6. Dislodge the cells and process or assay as desired.

### *3.7.3 Transduction of Mammalian Cells in Suspension*

Mammalian cells in suspension can be used when a larger number of transduced cells are required, such as for protein production [13]. A procedure is described here that is used to transduce a CHO-K1 suspension culture with BacMam. CHO-K1 suspension cells grow well in EX-CELL 302 medium (JRH Biosciences) and are usually split every 3–4 days.

1. Seed 100 mL medium with CHO-K1 cells at $5 \times 10^5$ cells/mL in a 500-mL shake flask. Incubate overnight at 37 °C, 5 % $CO_2$ with shaking (120 rpm).

2. Add sufficient virus stock to obtain an MOI of approximately 15 and sodium butyrate to 5 mM, continue shaking for 24–48 h. A histone deacetylase inhibitor (e.g., sodium butyrate or trichostatin A) can be used to enhance transduction efficiency [9].
3. Harvest the cells and process or assay as desired.

## 4 Notes

1. A useful feature of the pFastBac1 and derivative vectors is the gentamicin resistance marker, in addition to the ampicillin resistance marker. The gentamicin resistance marker provides an additional antibiotic selection for the transfer vectors. Given that most of the commonly used plasmid vectors lack this marker, transfer of a restriction fragment from these vectors to pFastBac1 vectors can be simplified by using a "shotgun" approach. This can be done by directly ligating a restriction-digested donor plasmid DNA with a properly prepared pFastBac1 vector DNA, and selecting the recombinants on plates containing both ampicillin and gentamicin following transformation. This approach eliminates the need to gel purify the restriction fragment from its vector. The gentamicin resistance in the pFastbac1 backbone is prone to loss by rearrangement because of sequence duplication near the translation start so we have found it useful to maintain selective pressure during the transfer vector construction.
2. The DH10Bac transformants can also be incubated at 37 °C for >24 h. We have found that some constructs are inhibitory to DH10bac cells when grown at 37 °C on selective medium. In *E. coli* there are two cryptic transcripts originating in the hCMV-IE1 promoter region [34] and the low level expression of some of the recombinant genes has on rare occasions made BacMam transfer vector generation problematic. We occasionally experience similar cDNA dependent toxicity with conventional mammalian expression vector constructs, but the issue is more significant in DH10bac cells because triple antibiotic selection is required for bacmid generation. Use of mammalian active promoters other than the hCMV-IE1 promoter may be less problematic. We also have a low copy number variant of the BacMam transfer vector in an attempt to minimize the levels of the background transcript.

   Because it is difficult to accurately predict which constructs may be problematic, we generally reduce the growth temperature to 30 °C and minimize the concentrations of the required antibiotics. It is important to use only the largest white colonies as small white colonies with aberrant transpositions may

sometimes occur. It is essential to restreak the colonies on DH10Bac plates to ensure uniformity. Even though the colony appears white, it is possible that it is a mixture of colonies comprising recombinant and nonrecombinant bacmids.

3. Some protocols recommend incubation for up to 24 h at 37 °C in DH10Bac culture medium (LB broth with 50 μg/mL kanamycin, 10 μg/mL tetracycline, and 7 μg/mL gentamicin). We found that in some cases cells grown under these conditions appear to clump or lyse.
4. Sf-9 cultures are easy to establish from a frozen stock. A vial of frozen cells is quickly thawed by hand warmth and transferred to 10 mL of HyClone SFX in a 125 mL vented shake flask. We use a biosafety cabinet for maintaining sterility when handling cultures. The new culture is incubated at 27 °C on a shaker at 80 rpm. After a few days, the cells are split to $10^6$ cells/mL. Infected and uninfected Sf-9 cultures are incubated in the same enclosed shakers.
5. We have found that Sf-9 cells cultured in HyClone SFX or Ex-Cell 420 will remain a homogenous culture more than 6 months if they are routinely split 1:15 every 3–4 days. With some other serum free formulations we tested the cell population changes with passage, thereby requiring more frequent replacement with a culture from frozen stocks.
6. We prefer individual T-flasks for Sf-9 transfections because each virus is well isolated from others generated on the same day, and cross contamination is avoided. In addition, compared to six-well tissue culture plates, T-25 flasks have a larger surface area, and a larger volume of P0 can be produced.
7. The medium supplemented with FBS may be added any time after 4 h, but it is normally added the next day. Transfections with FuGENE HD can be performed in the presence of FBS so no media change is necessary.
8. Addition of 5 % FBS is required for long-term stability of virus stocks. We routinely add the FBS at the time of infection for the sake of simplicity. We have also noted higher virus titers when the FBS is present in the insect cell culture medium during amplification. For some applications it may be necessary to omit FBS to reduce background levels in subsequent assays using transduced mammalian cells (*see* **Note 11** as well).
9. The appearance of the lawn at P0 harvest is an indicator of the relative titer of the virus stock, e.g., minimal lysis suggests a low titer. We do not routinely determine titers for P0 stocks. Titer yields for the P1 stock will exceed $5 \times 10^8$ pfu/mL for a fairly broad range of input P0 titers. We prefer to error on the side of employing a lower virus inoculum for the reasons outlined below (*see* **Note 10**). For some constructs significant cell

lysis occurs and the conditioned medium will be slightly cloudy. Conditioned medium containing virus is visually less clear than medium straight from the bottle; a large cell pellet and very clear virus stock may indicate that the infection and amplification did not go well.

10. An MOI of <1 is desired to reduce the production of defective virus particles. Some labs establish a defined MOI, for example, 0.25, as a target for amplification. If the MOI is too low, then the cell numbers during amplification can rise above the desired limit for the culture medium and insect cell line in use ($6–10 \times 10^6$ cells/mL). Virus yields can be maximized ($\sim 10^9$ pfu/mL) by optimizing the infection conditions to allow the cells to reach a maximum infected cell count (for HyClone, $<10^7$ cells/mL).
11. Conditioned insect cell medium is complex, and it may be desirable to transfer the virus to a defined buffer to simplify downstream assays. Selected functional GPCR assays can be sensitive to components in the conditioned insect cell medium. In addition, some neuronal cell lines are sensitive to glutamate found in the medium. There are several approaches to resolve this issue. The virus can be concentrated and resuspended in PBS or in a standard mammalian cell culture medium. Equipment required can be as simple as a centrifuge to pellet the virus or as sophisticated as cross-flow filtration equipment. These procedures are beyond the scope of this chapter and in most instances are not required as most mammalian cell lines can tolerate media mixtures containing up to 25 % conditioned insect cell media.
12. Cell culture methods vary between labs and the preferred medium for a particular cell line may not be optimal for BacMam transduction. Although our approach for BacMam transductions is to make only minimal changes to the culture conditions for the mammalian cell lines, it has become apparent that certain media can adversely affect transduction efficiency [38–40]. BacMams can be used for transductions without knowing the titer [41], but we routinely determine the virus titer prior to mammalian cell transductions.
13. Examples of BacMam transducible cells can be found in the literature [2, 5, 6, 8, 9, 27, 28, 32, 40–42]. Discrepancies in transduction efficiency are observed among isogenic cell lines derived from different lineage or sources. For example, a freshly isolated clonal CHO-K1 cell line derived in our laboratory appears to be more susceptible to BacMam transduction compared to a "standard" laboratory CHO-K1 line, and one HEK-293 cell line may perform better than another line from a different lineage (unpublished observations).

14. We and others have used cells transduced with BacMams as replacements for stable cell lines for various applications [11–13, 15–17, 42, 43]. Detailed methods to optimize cell based assays using BacMam viruses were recently published [44]. For functional assays in BacMam transduced cells an important negative control is a BacMam virus generated using an empty BacMam transfer vector that does not express a recombinant gene.
15. For some assays, such as antibody staining of extracellular epitopes, we prefer to harvest the cells using Versene rather than trypsin. If the protein of interest is intracellular or if cells will be replated and assayed the next day, then trypsin can safely be substituted to improve the yield of cells.

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# Part III

## Insect Cell Culture

# Chapter 6

# Available Lepidopteran Insect Cell Lines

**Dwight E. Lynn and Robert L. Harrison**

## Abstract

This chapter lists the known cell lines from Lepidoptera, largely based on previous compilations of insect cell lines published by W. Fred Hink. More than 320 lines from 65 species are listed. The official designation is given for each cell line as well as the species, tissue source, and, when known, the susceptibilities to baculoviruses.

**Key words** Lepidoptera, Continuous cell lines

## 1 Introduction

Early in the history of insect cell culturing, researchers in the field began meeting at 3–4-year intervals at International Conferences on Invertebrate Tissue Culture. The first of these was held in Montpellier, France, in 1962, which, perhaps not coincidentally, was the year that the first continuous insect cell lines were described in the literature [1]. In the 1970s and 1980s, W. Frederick Hink prepared compilations of insect cell lines [2–6] that were included in the proceedings from several of the subsequent International Conferences. His lists form the backbone of the listing included in this chapter (Table 1). The previous version of this chapter [7] included cell lines developed in the 15 years after Hink's last compilation and these tables have been updated to include the past five years. Table 1 now includes more than 320 cell lines from various lepidopteran species, providing a vast supply of material for research on baculoviruses. Analyzing the information from the table shows that lepidopteran cell line availability has steadily increased at about 50 new lines per decade (Fig. 1).

While we have not made an extensive literature search on virus susceptibilities of these cell lines (most of the details on viruses included in Table 1 were reported in the original publication

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**Table 1**
**Lepidopteran cell lines**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| *Adoxophyes orana* | Ao/I | Adult ovaries | AcMNPV, AdorMNPV, MbMNPV, SfMNPV | **NP-1** |
| *Adoxophyes orana fasciata* | FTRS-AoL1 | Neonate larvae | AdorMNPV, PlxyMNPV | [9] |
| | FTRS-AoL2 | Neonate larvae | AdorMNPV, PlxyMNPV | [9] |
| *Adoxophyes sp.* | FTRS-AfL | Neonate larvae | AdorMNPV, PlxyMNPV | [9] |
| *Agrotis ipsilon* | AiE1611T | Embryos | AcMNPV, AnfaNPV, AgMNPV, AgipMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV | [10] |
| | AiEd6T | Embryos | AgipMNPV | [10] |
| | BCIRL/AMCY-AiOV-CLG | Adult ovaries and fat body | PlxyMNPV | [11] |
| | BCIRL/AMCY-AiTS-CLG | Adult testes and fat body | | [11] |
| *Amyelois transitella* | HCRL-ATO10 | Pupal ovaries | AcMNPV | [13] |
| | HCRL-ATO20 | Pupal ovaries | AcMNPV | [12] |
| *Anagrapha falcifera* | BCIRL/AMCY-AfOV-CLG | Adult ovaries and fat body | AcMNPV | [11] |
| | BCIRL/AMCY-AfTS-CLG | Adult testes and fat body | | [11] |
| *Antheraea eucalypti* | | Pupal ovaries | BmNPV | [1] |
| | RML-2 subline of Grace's *A. eucalypti* cells | Pupal ovaries | | [1] |
| *Antheraea pernyi* | | Pupal ovaries | | **NP-2** |
| | NISES-AnPe-426 | Embryos | | [13] |
| | NISES-AnPe-428 | Embryos | AnyaNPV | [14] |
| *Antheraea yamamai* | | Pupal ovaries | | [15] |
| *Anticarsia gemmatalis* | BCIRL/AMCY-AgE-CLG | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-AgOV-CLG1 | Adult ovaries and fat body | AgMNPV | [11] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | BCIRL/AMCY-AgOV-CLG2 | Adult ovaries and fat body | AcMNPV, AgMNPV | [11] |
| | BCIRL/AMCY-AgOV-CLG3 | Adult ovaries and fat body | AcMNPV, AgMNPV | [11] |
| | UFL-AG-286 | Embryos | AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV | [16] |
| *Archippus breviplicanus* | FTRS-AbL81 | Neonate larvae | | [9] |
| *Bombyx mandarina* | NIAS-Boma-529b | Larval fat body | BmNPV | [17] |
| | SES-Bma-O1A | Mature embryos | | [18] |
| | SES-Bma-O1R | Mature embryos | | [18] |
| *Bombyx mori* | Bm-N | | BmNPV | **NP-3** |
| | | Larval midguts | | [19] |
| | Bm5 | Larval ovaries | | [20] |
| | Bm-21E-HNU5 | Embryos | AcMNPV, ArNPV, HearMNPV, PlxyGV | [21] |
| | Bm-Em-1 | Embryos | BmNPV | [22] |
| | DZNU-Bm-1 | Larval ovaries | BmNPV | [23] |
| | DZNU-Bm-12 | Larval ovaries | BmNPV | [24] |
| | NISES-BoMo-MK | Embryos | | [25] |
| | NISES-BoMo-KG | Embryos | | [25] |
| | NISES-BoMo-DZ | Embryos | | [25] |
| | NISES-BoMo-OH | Embryos | | [25] |
| | NIV-BM-1296 | Larval ovaries | AcMNPV, BmNPV | [26] |
| | NIV-BM-197 | Pupal ovaries | AcMNPV, BmNPV | [26] |
| | SES-Bm-1 30A | Mature embryos | | [18] |
| | SES-Bm-1 30R | Mature embryos | | [18] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | SES-Bm-e 21A | Mature embryos | | [18] |
| | SES-Bm-e 21B | Mature embryos | | [18] |
| | SES-Bm-e 21R | Mature embryos | | [18] |
| | SES-BoMo-15A | Embryos | BmNPV | [27] |
| | SES-BoMo-C129 | Embryos | BmNPV | [28] |
| | SES-BoMo-JI25 | Embryos | | [28] |
| | SPC-Bm36 | Pupal ovaries | | **NP-4** |
| | SPC-Bm40 | Pupal ovaries | | **NP-4** |
| *Buzura suppressaria* | WIV-BS-481 | Larval hemocytes | | **NP-5** |
| | WIV-BS-484 | Imaginal ovaries | BusuNPV | **NP-5** |
| *Cactoblastis cactorum* | BCIRL-Cc-AM | Adult ovaries | AcMNPV, GmMNPV | [29] |
| | BCIRL-Cc-JG | Adult ovaries | AcMNPV, GmMNPV | [29] |
| *Chilo suppressalis* | | Larval hemocytes | | [30] |
| *Choristoneura fumiferana* | FPMI-CF-1 | Midguts | | [31] |
| | FPMI-CF-2 | Midguts | | [31] |
| | FPMI-CF-3 | Midguts | | [31] |
| | FPMI-CF-203 | Midguts | | [31] |
| | FPMI-CF-50 | Pupal ovaries | AcMNPV, CfMNPV | **NP-6** |
| | FPMI-CF-60 | Pupal ovaries | AcMNPV, CfMNPV | **NP-6** |
| | FPMI-CF-70 | Pupal ovaries | AcMNPV, CfMNPV | **NP-6** |
| | IPRI-CF-1 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-CF-10 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-CF-12 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-Cf124 | Larvae | CfMNPV | [32] |
| | IPRI-CF-16 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-CF-16T | Neonate larvae | | **NP-6** |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | IPRI-CF-5 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-CF-6 | Neonate larvae | CfMNPV | **NP-6** |
| | IPRI-CF-8 | Neonate larvae | CfMNPV | **NP-6** |
| *Choristoneura occidentalis.* | | Embryos, neonate larvae, and ovaries | CfMNPV | **NP-6** |
| *Chrysodeixis chalcites* | WU-CcE-1 | Embryos | ChchNPV, TnSNPV | [33] |
| *Chrysodeixis (Pseudoplusia) includens* | UGA-CiE1 | Embryos | AcMNPV | [34] |
| *Clostera anachoreta* | CAF-Clan I | Embryos | AcMNPV | [35] |
| | CAFClan II | Embryos | AcMNPV, EcobNPV | [35] |
| *Cydia pomonella* | CP-1268 | Embryos | | [36] |
| | CP-169 | Embryos | | [36] |
| | CpDW1 | Embryos | | [37] |
| | CpDW2 | Embryos | | [37] |
| | CpDW3 | Embryos | | [37] |
| | CpDW4 | Embryos | | [37] |
| | CpDW5 | Embryos | | [37] |
| | CpDW6 | Embryos | | [37] |
| | CpDW9 | Embryos | | [37] |
| | CpDW10 | Embryos | | [37] |
| | CpDW11 | Embryos | | [37] |
| | CpDW12 | Embryos | | [37] |
| | CpDW13 | Embryos | | [37] |
| | CpDW14 | Embryos | CpGV | [37] |
| | CpDW15 | Embryos | CpGV | [37] |
| | 200 “primary” cell lines | Embryos and larval hemocytes | ChmuNPV, CpGV | [38] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | IZD-Cp 4/13 | | | [39] |
| | IZD-CP1508 | Larval hemocytes | | **NP-7** |
| | IZD-CP2202 | Larval hemocytes | | **NP-7** |
| | IZD-CP2507 | Larval hemocytes | | **NP-7** |
| | IZD-CP0508 | Larval hemocytes | | **NP-7** |
| *Danaus plexippus* | DpN1 | Embryos | AcMNPV | [40] |
| | BCIRL-DP-AM/JG | Adult ovaries | AcMNPV, AgMNPV, AnfaMNPV, PlxyMNPV | [41] |
| *Ectropis obliqua* | SIE-EO-801 | Pupal ovaries | | [42] |
| | SIE-EO-803 | Pupal ovaries | | [42] |
| *Ephestia kuehniella* | IPLB-Ekx4T | Embryos | AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV | [43] |
| | IPLB-Ekx4V | Embryos | AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV | [43] |
| *Epiphyas postvittana* | EpN1.10 | Neonate larvae | EppoNPV | [44] |
| *Estigmene acrea* | EA1174A (=BTI-EAA) | Larval hemocytes | AcMNPV | [45] |
| | EA1174H | Larval hemocytes | AcMNPV | [45] |
| *Eurema hecabe* | NTU-YB | Pupae | | [46] |
| *Euxoa scandens* | IAFEs-1 | Ovaries | AcMNPV, BmNPV, GmMNPV, DiwaNPV | [47] |
| *Galleria mellonella* | | Ovaries | | [48] |
| | GaMe-LF1 | Larval fat body | | [49] |
| *Gnorimoschema operculella* | G01-874 | Embryos | | [50] |
| | PTM | Embryos | | **NP-8** |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| *Helicoverpa armigera* | BCIRL-HA-AM1 | Pupal ovaries | AgMNPV | [51] |
| | CSIRO-BCIRL-HA1 | Ovaries | AcMNPV, HzSNPV | [52] |
| | CSIRO-BCIRL-HA2 | Ovaries | HzSNPV | [52] |
| | CSIRO-BCIRL-HA3 | Ovaries | HzSNPV | [52] |
| | HaEpi | Larval integument | | [53] |
| | IOZCAS-Ha-I | Larval fat body | HearMNPV | [54] |
| | KU-HaEmb1 | Embryos | SeMNPV | [55] |
| | KU-HaEmb2 | Embryos | AcMNPV, HearSNPV, SeMNPV | [55] |
| | KU-HaPO1 | Pupal ovaries | AcMNPV, HearSNPV, SeMNPV | [55] |
| | KU-HaPO2 | Pupal ovaries | AcMNPV, HearSNPV, SeMNPV | [55] |
| | KU-HaAO1 | Adult ovaries | HearSNPV | [55] |
| | NIV-HA-197 | Embryo | AcMNPV, HearSNPV, SpltMNPV | [56] |
| *Helicoverpa punctigera* | CSIRO-BCIRL-HP1 | Embryos | AcMNPV, HzSNPV | [52] |
| | CSIRO-BCIRL-HP2 | Embryos | AcMNPV, HzSNPV | [52] |
| | CSIRO-BCIRL-HP3 | Embryos | AcMNPV, HzSNPV | [52] |
| | CSIRO-BCIRL-HP4 | Ovaries | AcMNPV, HzSNPV | [52] |
| | CSIRO-BCIRL-HP5 | Ovaries | AcMNPV, HzSNPV | [52] |
| *Helicoverpa zea* | BCIRL/AMCY-HzE-CLG1 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-HzE-CLG2 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-HzE-CLG3 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-HzE-CLG5 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-HzE-CLG6 | Embryos | AcMNPV | [11] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | BCIRL/AMCY-HzE-CLG7 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-HzE-CLG8 | Embryos | | [11] |
| | BCIRL/AMCY-HzE-CLG9 | Embryos | | [11] |
| | BCIRL-HZ-AM1 | Pupal ovaries | HzSNPV, HearSNPV | [51] |
| | BCIRL-HZ-AM2 | Pupal ovaries | HzSNPV | [51] |
| | BCIRL-HZ-AM3 | Pupal ovaries | HzSNPV | [51] |
| | IMC-HZ-1 | Adult ovaries | HzSNPV | [57] |
| | IPLB-HZ-1074 | Pupal ovaries | HzMNPV | [58] |
| | IPLB-HZ-1075 | Pupal ovaries and fat body | HzMNPV | [58] |
| | IPLB-HZ-1079 | Fat body | HzMNPV | [58] |
| | IPLB-HZ-110 | Pupal ovaries | HzMNPV | [58] |
| | IPLB-HZ-124Q | Pupal ovaries | HzMNPV | [58] |
| | RP-HzVNC-AW1 | Larval ventral nerve cord | | [59] |
| | RP-HzGUT-AW1 | Larval midgut | | [59] |
| | RP-HzOV-AW2 | Adult ovaries | | [59] |
| *Heliothis virescens* | BCIRL/AMCY-HvE-CLG1 | Embryos | AcMNPV, AgMNPV PlxyMNPV | [11] |
| | BCIRL/AMCY-HvE-CLG2 | Embryos | AcMNPV, AgMNPV PlxyMNPV | [11] |
| | BCIRL/AMCY-HvE-CLG3 | Embryos | AcMNPV, AgMNPV | [11] |
| | BCIRL/AMCY-HvOV-CLG | Adult ovaries | AcMNPV, AgMNPV, PlxyMNPV | [11] |
| | BCIRL/AMCY-Hv-TS-GES | Larval testes | AcMNPV | [11] |
| | BCIRL-HV-AM1 | Pupal ovaries | AcMNPV, AgMNPV, HzSNPV | [60] |
| | BCIRL-HV-AM2 | Pupal ovaries | AcMNPV, HzSNPV | [60] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | BCIRL/ RP-HvE-CLG1 | Embryos | | [59] |
| | BCIRL/ RP-HvE-CLG4 | Embryos | | [59] |
| | BCIRL/ RP-HvE-CLG5 | Embryos | | [59] |
| | BCIRL/ RP-HvE-CLG6 | Embryos | | [59] |
| | BCIRL/ RP-HvE-CLG7 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN2 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN3 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN11 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN12 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN14 | Embryos | | [59] |
| | BCIRL/ RP-HvE-HN16 | Embryos | | [59] |
| | BCIRL/ RP-HvVNC-WG1 | Larval ventral nerve cord | | [59] |
| | BCIRL/ RP-HvVNC-WG2 | Larval ventral nerve cord | | [59] |
| | BCIRL/ RP-HvVNC-WG3 | Larval ventral nerve cord | | [59] |
| | IPLB-HvE1a | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV | [61] |
| | IPLB-HvE1-lt | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, OpMNPV, RoMNPV | [61] |
| | IPLB-HvE1s | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV | [61] |
| | IPLB-HvE6a | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV | [61] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | IPLB-HvE6a-lt | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, OpMNPV, PlxyMNPV, RoMNPV | [61] |
| | IPLB-HvE6s | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV | [61] |
| | IPLB-HvE6s-lt | Embryos | AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, RoMNPV | [61] |
| | IPLB-HvT1 | Larval testicular sheath | AcMNPV, HzSNPV | [62] |
| *Homona magnanima* | FTRS-HmL45 | Neonate larvae | PlxyMNPV | [9] |
| *Hoshinoa longicellana* | FTRS-HlL1 | Neonate larvae | | [9] |
| | FTRS-HlL2 | Neonate larvae | | [9] |
| *Latoia viridissima* | | Larval hemolymph | LaviNPV | [63] |
| *Leucania separata* | NIAS-LeSe-11 | Larval fat body (female) | AcMNPV | [64] |
| *Lymantria dispar* | IPLB-LD-64 | Pupal ovaries | AcMNPV | [65] |
| | IPLB-LD-65 | Pupal ovaries | LdMNPV | [65] |
| | IPLB-LD-66 | Pupal ovaries | | [65] |
| | IPLB-LD-67 | Pupal ovaries | LdMNPV | [65] |
| | IPLB-LdEG | Embryos | AcMNPV, LdMNPV | [62] |
| | IPLB-LdEI | Embryos | AcMNPV, LdMNPV | [62] |
| | IPLB-LdEIt | Embryos | AcMNPV, LdMNPV | [62] |
| | IPLB-LdEp | Embryos | AcMNPV, LdMNPV | [62] |
| | IPLB-LdFB | Larval fat bodies | LdMNPV | [62] |
| | IZD-LD1307 | Larval testes | | **NP-9** |
| | IZD-LD1407 | Larval testes | AcMNPV | **NP-9** |
| | SCLd 135 | Ovaries | BmNPV, GmMNPV | **NP-9** |
| *Lymantria xylina* | NTU-LY-1 | Pupae | LdMNPV, LyxyMNPV, PenuMNPV | [66] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | NTU-LY-2 | Pupae | LdMNPV, LyxyMNPV, PenuMNPV | [66] |
| | NTU-LY-3 | Pupae | LdMNPV, LyxyMNPV, PenuMNPV | [66] |
| | NTU-LY-4 | Pupae | LdMNPV, LyxyMNPV, PenuMNPV | [66] |
| *Malacosoma disstria* | IPRI 108 | Larval hemocytes | AcMNPV, CfMNPV, LafisoNPV | [67] |
| | UMN-MDH-1 | Hemocytes of fifth-instar larvae | MadiNPV | **NP-10** |
| *Mamestra brassicae* | HPB-MB | Adult ovaries | AcMNPV, TnSNPV | **NP-11** |
| | IZD-MB0503 | Larval hemocytes | AcMNPV, MbMNPV | [68] |
| | IZD-MB0504 | Larval hemocytes | AcMNPV | [68] |
| | IZD-MB1203 | Larval ovary and dorsal vessel | AcMNPV | [68] |
| | IZD-MB2006 | Larval hemocytes | | [68] |
| | IZD-MB2007 | Larval hemocytes | | [68] |
| | IZD-MB2506 | Larval hemocytes | AcMNPV, MbMNPV | [68] |
| | MB-H 260 | Hemocytes | MbMNPV | [69] |
| | MB-H 260 | Hemocytes | MbMNPV | [69] |
| | MbL-3 | Neonate larvae | MbMNPV | [70] |
| | MbL-3 | Neonate larvae | MbMNPV | [70] |
| | NIAS-MaBr-85 | Larval fat body (male) | AcMNPV | [71] |
| | NIAS-MaBr-92 | Larval hemocytes | AcMNPV | [72] |
| | NIAS-MaBr-93 | Larval hemocytes | AcMNPV | [72] |
| | NIAS-MB-19 | Pupal ovaries | | [73] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | NIAS-MB-25 | Pupal ovaries | | [73] |
| | NIAS-MB-32 | Pupal ovaries | | [73] |
| | SES-MaBr-1 | Larval fat body | AcMNPV | [74] |
| | SES-MaBr-2 | Larval fat body | AcMNPV | [74] |
| | SES-MaBr-3 | Larval fat body | AcMNPV | [74] |
| | SES-MaBr-4 | Larval fat body | AcMNPV | [74] |
| | SES-MaBr-5 | Larval fat body | AcMNPV | [74] |
| *Manduca sexta* | FPMI-MS-12 | Neonate larvae | AcMNPV | **NP-6** |
| | FPMI-MS-4 | Neonate larvae | AcMNPV | **NP-6** |
| | FPMI-MS-5 | Neonate larvae | AcMNPV | **NP-6** |
| | FPMI-MS-7 | Neonate larvae | AcMNPV | **NP-6** |
| | MRRL-CH-1 | Embryos | | [75] |
| | MRRL-CH-2 | Embryos | | [75] |
| *Maruca vitrata* | NTU-MV | pupal ovary | | [76] |
| *Mythimna convecta* | BPMNU-MyCo-1 | Fat body | | [77] |
| *Mythimna (Leucania) separata* | NEAU-Ms-980312 | Embryos | MyseNPV | [78] |
| *Orgyia leucostigma* | IPLB-OlE505A | Embryos | AcMNPV, AnfaMNPV, OpMNPV, OrleNPV, RoMNPV | [79] |
| | IPLB-OlE505s | Embryos | OpMNPV, OrleNPV | [79] |
| | IPLB-OlE7 | Embryos | OpMNPV, OrleNPV | [79] |
| | IPRI-OL-12 | Neonate larvae | OpMNPV, OpSNPV | [80] |
| | IPRI-OL-13 | Neonate larvae | OpMNPV, OpSNPV | [80] |
| | IPRI-OL-4 | Neonate larvae | OpMNPV, OpSNPV | [80] |
| | IPRI-OL-9 | Neonate larvae | OpMNPV, OpSNPV | [80] |
| *Ostrinia nubilalis* | AFKM-On-H | Larval hemocytes | AcMNPV, AnyaNPV | [81] |
| | BCIRL/AMCY-OnFB-GES1 | Larval fat body | | [11] |
| | BCIRL/AMCY-OnFB-GES2 | Larval fat body | | [11] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | UMC-OnE | Embryos | AcMNPV, PlxyMNPV | [82] |
| *Pandemis heparana* | FTRS-PhL | Neonate larvae | PlxyMNPV | [9] |
| *Papilio xuthus* | Px-58 | Pupal ovaries | | [83] |
| | Px-64 | Pupal ovaries | | [83] |
| | RIRI-PX1 | Neonate larvae | AcMNPV | [84] |
| *Phthorimaea operculella* | NIV-PTM-1095 | Embryos | | [85] |
| | ORS-Pop-93 | Embryos | AcMNPV, SpliNPV | [86] |
| | ORS-Pop-95 | Embryos | PhopGV, SpliGV | [86] |
| *Pieris rapae* | BTI-PR10B | Embryos | | [87] |
| | BTI-PR8A1 | Embryos | | [87] |
| | BTI-PR8A2 | Embryos | | [87] |
| | BTI-PR9A | Embryos | | [87] |
| | NIAS-PRC-819A | Ovaries | | [88] |
| | NIAS-PRC-819B | Ovaries | | [88] |
| | NIAS-PRC-819C | Ovaries | | [88] |
| | NYAES-PR4A | Embryos | | [87] |
| *Plodia interpunctella* | IAL-PID2 | Imaginal wing discs | AgMNPV | [89] |
| | IPLB-PiE | Embryos | | **NP-12** |
| | UMN-PIE-1181 | Embryos of a malathion-resistant strain | | [90] |
| *Plutella xylostella* | BCIRL/AMCY-PxE-CLG | Embryos | AcMNPV, PlxyMNPV | [11] |
| | BCIRL/AMCY-PxLP-CLG | Larvae/pupae (whole insects) | AcMNPV, PlxyMNPV | [11] |
| | IPLB-PxE1 | Embryos | AcMNPV, AnfaMNPV, RoMNPV | **NP-12** |
| | IPLB-PxE2 | Embryos | AcMNPV, AnfaMNPV, RoMNPV | **NP-12** |
| | PX-1187 | Embryos | | [91] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | BCIRL-PX2-HNU3 | | AcMNPV, ArGV, ArNPV, HearMNPV | [92] |
| *Pseudaletia unipuncta* | BTI-Pu-2 | Embryos | AcMNPV | [93] |
| | BTI-Pu-A7 | Embryos | AcMNPV | [93] |
| | BTI-Pu-A7S | Embryos | AcMNPV | [93] |
| | BTI-Pu-B9 | Embryos | AcMNPV | [93] |
| | BTI-Pu-M | Embryos | AcMNPV | [93] |
| | BTI-Pu-M1B | Embryos | AcMNPV | [93] |
| *Samia cynthia* | Several lines | Pupal hemocytes | | [94] |
| *Spilarctia seriatopunctata.* | NIAS-SpSe-1 | Larval fat body (male) | | [95] |
| *Spilosoma imparilis* | FRI-SpIm-1229 | Larval fat bodies | HycuNPV, SpimNPV | [96] |
| *Spodoptera exigua* | BCIRL/AMCY-SeE-CLG1 | Embryos | | [11] |
| | BCIRL/AMCY-SeE-CLG4 | Embryos | | [11] |
| | BCIRL/AMCY-SeE-CLG5 | Embryos | | [11] |
| | IOZCAS-Spex-II | Larval fat body | SeMNPV | [97] |
| | IOZCAS-Spex-III | Larval fat body | SeMNPV | [97] |
| | IOZCAS-Spex XI | Pupal ovaries | AcMNPV, SeMNPV, SpltNPV | [98] |
| | IOZCAS-Spex 12 | Pupal ovaries | AcMNPV, SeMNPV, SpltNPV | [99] |
| | Se3FH | Neonate larvae | SeMNPV | [100] |
| | Se4FH | Neonate larvae | SeMNPV | [100] |
| | Se5FH | Neonate larvae | SeMNPV | [100] |
| | Se6FHA | Neonate larvae | SeMNPV | [100] |
| | Se6FHB | Neonate larvae | SeMNPV | [100] |
| | SeHe920-1a | Hemocytes | | [101] |
| | UCR-SE-1 | Neonate larvae | AcMNPV, SeMNPV | [102] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| *Spodoptera frugiperda* | BCIRL/AMCY-SfTS-GES | Larval testes | AcMNPV, PlxyMNPV | [11] |
| | IAL-SFD1 | Imaginal wing discs | AcMNPV, AgMNPV, TnSNPV | [89] |
| | IPLB-Sf1254 | Pupal ovary | AcMNPV, TnSNPV, SfMNPV | [103] |
| | IPLB-Sf21, IPLB-Sf21AE, Sf-9 | Pupal ovary | AcMNPV, PlxyMNPV, SfMNPV, SpliNPV, ThorNPV | [103] |
| *Spodoptera littoralis* | HPB-SL | Larvae | AcMNPV, TnSNPV | **NP-11** |
| | SPC-Sl-48 | Pupal ovaries | AcMNPV | [104] |
| | SPC-Sl-52 | Pupal ovaries | AcMNPV | [104] |
| | UIV-SL-373 | Pupal ovaries | AcMNPV, SeMNPV, SpliNPV, TnSNPV | [105] |
| | UIV-SL-573 | Pupal ovaries | AcMNPV, SeMNPV, SpliNPV, TnSNPV | [105] |
| | UIV-SL-673 | Pupal ovaries | AcMNPV, SeMNPV, SpliNPV, TnSNPV | [105] |
| *Spodoptera litura* | IBL-SL1A | Pupal ovaries | SpltNPV | [106] |
| | NIV-SU-893 | Pupal ovaries | | [107] |
| | NIV-SU-992 | Larval ovaries | | [108] |
| *Spodoptera ornithogalli* | BCIRL-503-HNU1 | Adult ovaries | | [109] |
| | BCIRL-504-HNU4 | Adult ovaries | | [109] |
| *Trichoplusia ni* | BCIRL/AMCY-TnE-CLG1 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-TnE-CLG1MK | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-TnE-CLG2 | Embryos | AcMNPV | [11] |

(continued)

**Table 1**
**(continued)**

| Species | Designation | Tissue source | Baculovirus infectivity[a] | References[b] |
|---|---|---|---|---|
| | BCIRL/AMCY-TnE-CLG2MK | Embryos | AcMNPV, PlxyMNPV | [11] |
| | BCIRL/AMCY-TnE-CLG3 | Embryos | AcMNPV | [11] |
| | BCIRL/AMCY-TnTS-GES1 | Larval testes | AcMNPV, PlxyMNPV | [11] |
| | BCIRL/AMCY-TnTS-GES3 | Larval testes | PlxyMNPV | [11] |
| | BTI-TN5B1-4(High Five®) | Embryos | AcMNPV, PlxyMNPV, ThorNPV, TnSNPV | [110] |
| | BTI-TN5C1 | Embryos | | [110] |
| | BTI-TN5F2 | Embryos | | [110] |
| | BTI-TN5G2A1 | Embryos | | [110] |
| | BTI-TN5G3 | Embryos | | [110] |
| | BTI-TN5G33 | Embryos | | [110] |
| | IAL-TND1 | Imaginal wing discs | AcMNPV, AnfaMNPV, AgMNPV, PlxyMNPV | [111] |
| | IPLB-TN-R[b] | 3-day-old embryos | AcMNPV, AnfaMNPV, AgMNPV, PlxyMNPV | [112] |
| | MSU-TnT4 | Embryos | AcMNPV | [113] |
| | QAU-BTI-Tn9-4s | Embryos | AcMNPV | [114] |
| | Several lines | Pupal ovary and fat body | TnSNPV | [115] |
| | TN-368 | Adult ovaries | AcMNPV, TnSNPV, GmMNPV, AgMNPV, PlxyMNPV | [116] |

[a]In most cases, the virus susceptibilities were reported in the original publication of the cell lines. In some cases, they are from the Hink compilations [2–6] or the Granados and Hashimoto review [8] as discussed in the Introduction

[b]NP: Not published. Most of these cell lines were reported in the Hink compilations [2–6]. The researcher(s) that communicated the cell line to Hink were as follows: NP-1: D. Peters, NP-2: H. Lee, NP-4: J.M. Quiot, NP-5: Xie Tianen, Wang Luming, and Liu Songhus, NP-6: S. S. Sohi, NP-7: U. Mahr and H. G. Miltenburger, NP-8: T. D. C. Grace, NP-9: H. G. Miltenburger, NP-10: K. R. Tsang, NP-11: I. Hilwig and F. Alapatt, NP-12: D. E. Lynn. NP-3: the *B. mori* Bm-N line is widely distributed and used with BmNPV but we have been unable to discern the original source (investigator or tissue of origin) of this line

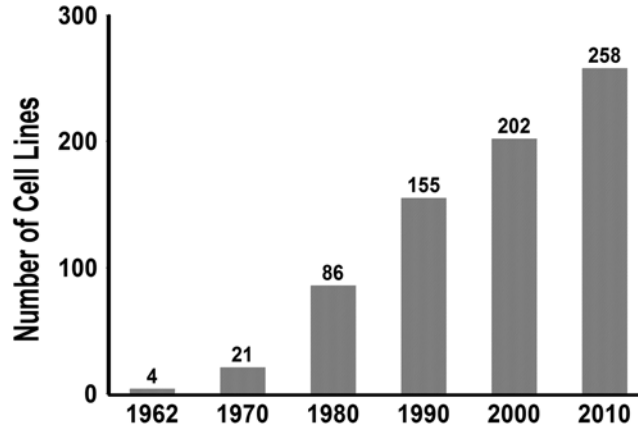


**Fig. 1** Number of cell lines reported from Lepidoptera based on the information in Table 1, accumulative by decade

describing the specific cell line or in one of Hink's compilations [2–6] or the Granados and Hashimoto review [8]), approximately 60 % of these cell lines are known to replicate one or more baculovirus. The virus designations used in Table 1 are based on the original source of the virus as defined in Table 2 which lists the baculoviruses that have been grown in insect cell culture. Note that a given baculovirus is named based on the insect species from which it was first isolated. Over 100 lines are known to replicate the *Autographa californica* multiple nucleopolyhedrovirus, which may surprise the majority of researchers using Sf-9, High Five, or Sf21AE cells with this virus as an expression vector.

The current availability of some of these cell lines is unknown. While a few insect cell lines are available through repositories (such as the American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA, or the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG), researchers interested in the use of most of these cell lines will need to obtain them from other laboratories. If at all possible, then this should be from the original source of the cells. Unfortunately, many of the earliest insect cell culturists are no longer active in research, making this somewhat problematic. However, the pool of researchers that have created new cell cultures is relatively small, so contacting one of the active researchers in the field will likely lead to a source if the cell line is still in existence.

**Table 2**
**Baculovirus isolates grown in cell culture**

| Name of virus isolate[a] | Designation |
|---|---|
| *Anagrapha falcifera* NPV | AnfaNPV |
| *Antheraea yamamai* NPV | AnyaNPV |
| *Anticarsia gemmatalis* MNPV | AgMNPV |
| *Antheraea yamamai* NPV | AnyaNPV |
| *Artogeia rapae* GV | ArGV |
| *Artogeia rapae* NPV | ArNPV |
| *Autographa californica* MNPV | AcMNPV |
| *Bombyx mori* NPV | BmNPV |
| *Buzura suppressaria* NPV | BusuNPV |
| *Choristoneura fumiferana* MNPV | CfMNPV |
| *Choristoneura murinana* NPV | ChmuNPV |
| *Chrysodeixis chalcites* NPV | ChchNPV |
| *Cydia pomonella* GV | CpGV |
| *Diapropsis watersii* NPV | DiwaNPV |
| *Ecotropis obliqua* NPV | EcobNPV |
| *Epiphyas postvittana* NPV | EppoNPV |
| *Galleria mellonella* MNPV | GmMNPV |
| *Helicoverpa armigera* NPV | HearNPV |
| *Helicoverpa zea* SNPV | HzSNPV |
| *Hyphantria cunea* NPV | HycuNPV |
| *Lambdina fiscellaria somniaria* NPV | LafiNPV |
| *Latoia viridissima* NPV | LaviNPV |
| *Lymantria dispar* MNPV | LdMNPV |
| *Lymantria xylina* MNPV | LyxyMNPV |
| *Malacosoma disstria* NPV | MadiNPV |
| *Mamestra brassicae* MNPV | MbMNPV |
| *Mythimna (Leucania) separata* NPV | MyseNPV |
| *Orgyia leucostigma* NPV | OrleNPV |
| *Orgyia pseudotsugata* MNPV | OpMNPV |
| *Orgyia pseudotsugata* MNPV | OpMNPV |

(continued)

**Table 2**
**(continued)**

| Name of virus isolate[a] | Designation |
|---|---|
| *Orgyia pseudotsugata* SNPV | OpSNPV |
| *Perina nuda* MNPV | PenuMNPV |
| *Phthorimaea operculella* GV | PhopGV |
| *Plutella xylostella* GV | PlxyGV |
| *Plutella xylostella* MNPV | PlxyMNPV |
| *Rachiplusia ou* MNPV | RoMNPV |
| *Spilosoma imparilis* NPV | SpimNPV |
| *Spodoptera exigua* MNPV | SeMNPV |
| *Spodoptera frugiperda* MNPV | SfMNPV |
| *Spodoptera littoralis* GV | SpliGV |
| *Spodoptera littoralis* NPV | SpliNPV |
| *Spodoptera litura* NPV | SpltNPV |
| *Thysanoplusia orichalcea* NPV | ThorNPV |
| *Trichoplusia ni* SNPV | TnSNPV |

[a]The viruses are *NPV* nucleopolyhedrovirus, *MNPV* multiple NPVs, *SNPV* single NPVs, *GV* granulovirus

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# Chapter 7

# Lepidopteran Insect Cell Line Isolation from Insect Tissue

**Dwight E. Lynn**

## Abstract

This chapter describes procedures for initiating new cell lines from lepidopteran larval tissues and embryos. The internal morphology is described along with methods for treating excised tissues and the primary cultures. Advice on culture medium and the tissues that will provide the best chance for new cell lines is discussed.

**Key words** Cell line establishment, Primary cultures, Internal morphology

## 1 Introduction

As shown in Chapter 6, established cell lines from Lepidoptera are widely available, with over 320 lines from 65 species. With such a diversity of material already available, most researchers working with insect viruses will not need to develop their own cell lines. On the other hand, many of the baculoviruses that have been discovered have never been grown in cell culture, so the possibility exists that efforts on a new virus may also require development of a new cell line. The methods described here are a collection of procedures that other researchers and I have found effective for initiating new cell lines. While virtually all insect stages may be used as a source of tissue for cell cultures, I will focus on larval and embryo tissues in this chapter with notes describing some other sources for insect cells.

## 2 Materials

### 2.1 Solutions

1. Commercial insect cell culture medium (*see* **Note 1**) supplemented with 5–10 % (v/v) fetal bovine serum and 5 μg/mL gentamicin sulfate (*see* **Note 2**).

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2. Divalent ion-free phosphate-buffered saline (PBS) for enzyme dissociation: 800 mg NaCl, 20 mg $KH_2PO_4$, 20 mg KCl, 150 mg $Na_2HPO_4 \cdot 7H_2O$, 23 mg $Na_2EDTA$ in demineralized water to 100 mL, then autoclaved or filter sterilized.
3. VMF trypsin (virus and mycoplasma free; cell culture tested).
4. 70 % ethanol.
5. Sterile (autoclaved) demineralized water.

### 2.2 Equipment

1. 35 mm tissue culture Petri dishes.
2. 12.5 or 25 $cm^2$ tissue culture flasks.
3. Laminar flow clean bench (*see* **Note 3**).
4. Dissecting tools, including two or more pairs of jeweler's forceps, micro scalpel, micro scissors (*see* **Note 4**).
5. Insect pins.
6. Small jar with a layer of cotton in the bottom containing 95 % ethanol (*see* **Note 5**).
7. Alcohol lamp with wind screen (*see* **Note 6**).
8. Sterile (autoclaved) glass Petri dish containing 4–5 mm thick paraffin (=wax dish).
9. Stereo dissecting microscope and light source.
10. 1 mL sterile pipets.
11. 250 μL pipettor.
12. Sterile pipet tips.
13. Plastic box, ~5 × 6 × 2 in. with airtight lid.

## 3 Methods

### 3.1 Disinfection

Most insects can withstand a short period in disinfecting solutions without significant harm. My preferred method is as follows:

1. Submerge the insect (or eggs if initiating embryo cultures) in 70 % ethanol for 5–20 min. The shorter time should be used with particularly fragile (e.g., thin-cuticled) insects and the longer time if the insect's typical environment is particularly dirty (*see* **Note 7**).
2. Rinse with at least two changes of sterile demineralized water. Several insects can be disinfected simultaneously and then held for 30–60 min in the final water rinse until needed. Keeping them submersed in this manner will result in a buildup of $CO_2$ from their natural respiration and this will act as an anesthetic to keep them immobile during the dissection.

**Fig. 1** Typical arrangement of equipment in the clean bench for dissections. The jar in the center contains ethanol and a small layer of cotton in the *bottom*. The dissecting tools are placed in the jar for at least 10 min, after which the alcohol is ignited by passing the tip over the alcohol lamp's flame (*see* **Note 9**). They can then be positioned on a small tray (in this case, it was fashioned from the container in which the microscalpel was purchased) until needed for the dissection

### *3.2 Dissection*

1. Place a dissecting microscope, alcohol lamp, and jar, and dissecting tools in the clean bench (Fig. 1) and turn on the airflow.
2. Wipe down the surface of the microscope with 70 % ethanol and place the dissecting tools in the alcohol jar for at least 10 min, then burn off the alcohol by briefly holding them over the lit alcohol lamp (*see* **Note 8**). Place them on the holding tray until needed.
3. Position the disinfected larva, dorsal side up, in a sterile wax dish. Insert an insect pin through the head and last abdominal segment of the larva and into the wax in the bottom of the dish (Fig. 2a, *see* **Note 9**).
4. Aseptically add enough culture medium to the dish to completely cover the insect.
5. Pinch the insect's cuticle with forceps and use the disinfected scissors to cut a small hole on the dorsal side of the next to last abdominal segment only deep enough to penetrate to the hemocoel.
6. Keeping the scissor blade parallel to the insect's body, cut the cuticle from tail to the first thoracic segment, taking care to not pierce the gut.

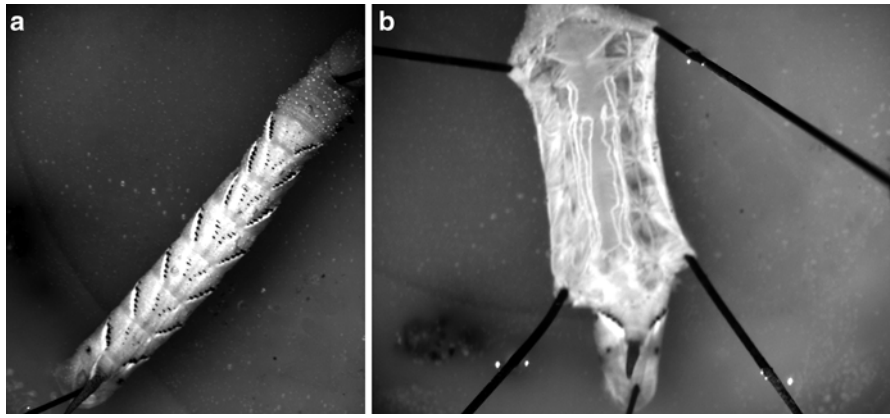


**Fig. 2** A fourth instar *Manduca sexta* larva pinned in a wax dish containing tissue culture medium. (**a**) Larva before first incision. (**b**) Larva with cuticle pinned to each side to expose internal organs and tissues

7. Use the forceps to grasp the cut edge and use either the scissors or another forceps to cut/tear the tracheoles that are connected to the gut. Once this is accomplished, more insect pins can be used on each side to hold open the incision (Fig. 2b).

### *3.3 Internal Morphology*

The internal morphology of lepidopteran larvae can be somewhat daunting to the uninitiated, but is actually fairly simple.

#### *3.3.1 Digestive Tract*

Since larval Lepidoptera are essentially eating machines, the most obvious structure is the digestive tract (Figs. 3, 4, and 5, FG and MG), which is divided into three morphologically and physiologically distinct sections—the foregut (FG), midgut (MG) and hindgut. Unless you specifically want to set up cultures from gut cells [1] (*see* **Note 10**), you should be especially cautious to avoid rupturing it.

#### *3.3.2 Malphigian Tubules*

Loosely connected to the midgut are the Malphigian tubules (Figs. 3, 4, and 5, MT), white tubes running along the digestive tract parallel to the body and often looped near the thorax and continuing back toward the posterior. These are the kidneys of the insect, responsible for removing nitrogenous wastes from the blood that are excreted into the digestive tract at the interface of the mid and hindguts. The uric acid crystals formed from the insect's nitrogenous waste creates their bright, refractive nature.

#### *3.3.3 Tracheals/ Tracheoles*

The tracheals/tracheoles are also quite apparent (Figs. 3 and 4, T). This is the respiratory system for insects and is a series of branching tubes that supply air to each tissue (*see* **Note 11**). They are typically very obvious, appearing somewhat silvery due to the refraction of the air they contain.

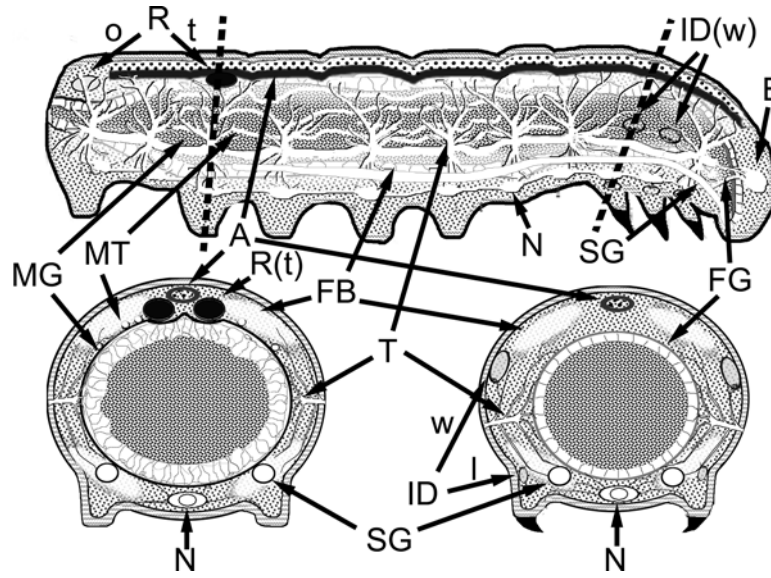


**Fig. 3** Diagrams of lepidopteran larva showing the locations of many tissues. *Top*: Lateral view. *Lower right*: Cross section through thorax. *Lower left*: Cross section through abdomen. *R* reproductive tissue (*o* ovaries, *t* testis), *ID* imaginal discs (*w* wings, *l* legs), *B* brain, *MG* midgut, *MT* Malphigian tubules, *A* dorsal aorta, *N* ventral nerve cord, *SG* salivary (silk) glands, *FG* foregut, *FB* fat body, *T* tracheoles

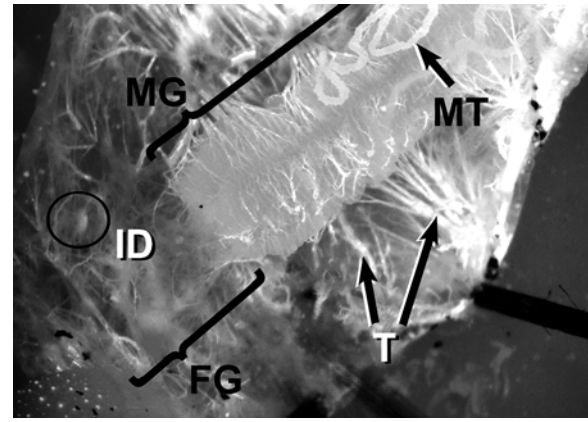


**Fig. 4** Thoracic and anterior abdomen of *M. sexta* larva. *ID* imaginal discs, *MG* midgut, *MT* Malphigian tubules, *FG* foregut, *T* tracheoles. The *circle* is the location of one of the imaginal discs. At this stage in the dissection, the lateral muscles largely obscure it, but the faint white mass in the middle of the circle is the mass of tracheoles that eventually become the wing venation in the adult

### 3.3.4 Fat Body

In the later stages of larval development, the fat body (physiologically equivalent to the mammalian liver and fat cells) becomes a prominent cell type. In most lepidopteran larvae, these occur in four bands of a relatively loose tissue (Fig. 3, FB), generally appearing bright white because of the large amount of lipids they contain.

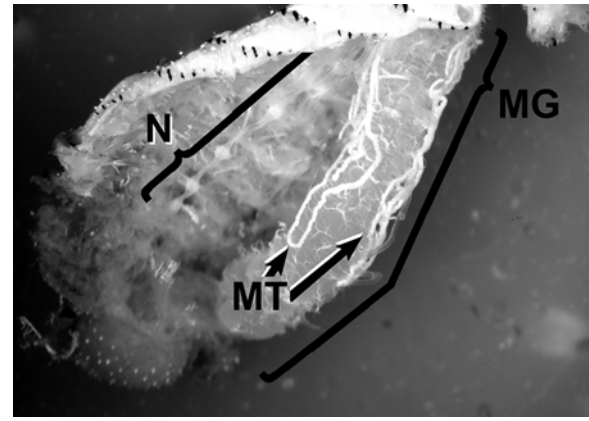


**Fig. 5** Thoracic and anterior abdomen of *M. sexta* larva showing a large portion of the abdomen with the midgut moved to the side revealing the ventral nerve cord (N). *MG* midgut, *MT* Malphigian tubules

*3.3.5 Nerve Cord*

After severing the tracheoles connected to the digestive tract, the gut can be gently stretched and moved to the side of the body. Doing so will reveal the ventral nerve cord (Figs. 3 and 5, N). This tissue, a white/light cream-colored structure, runs the length of the ventral side of the insect with enlarged areas (ganglia) in each segment.

*3.3.6 Salivary Glands*

The salivary glands are a pair of translucent tubes running from the head for about half to three quarters the length of the larva. These are usually on the ventral side of the digestive tract and slightly to each side, somewhat (albeit loosely) connected to the digestive tract (Fig. 3, SG).

*3.3.7 Dorsal Aorta*

Less apparent, because it typically is the same color as the cuticle and connective tissue, is the dorsal aorta, the insect's heart (Fig. 3, A). It is tightly connected to the insect cuticle and, depending on how near the midline you made your incision, you may have cut across this organ. However, it can often be identified by the regular muscle contractions.

*3.3.8 Reproductive Organs*

The reproductive organs will be in the abdomen, generally dorsal to the hindgut (in the case of ovaries) or the midgut (in the case of testes). Males typically have two ovoid testes (Fig. 3, R(t), Fig. 6c) that fuse into a single organ late in the last larval instar in some species. These can be brightly colored organs and, with species that have a lightly colored cuticle, can occasionally be seen through the cuticle as a means of identifying males from females (Fig. 6a, b). The ovaries in female larvae are typically a pair of cream-colored structures connected to a common oviduct and are smaller versions of the organs in the adults. These can be more difficult to identify in the larval stages because they typically remain quite small (contrary to the testes) until the pupal stage.

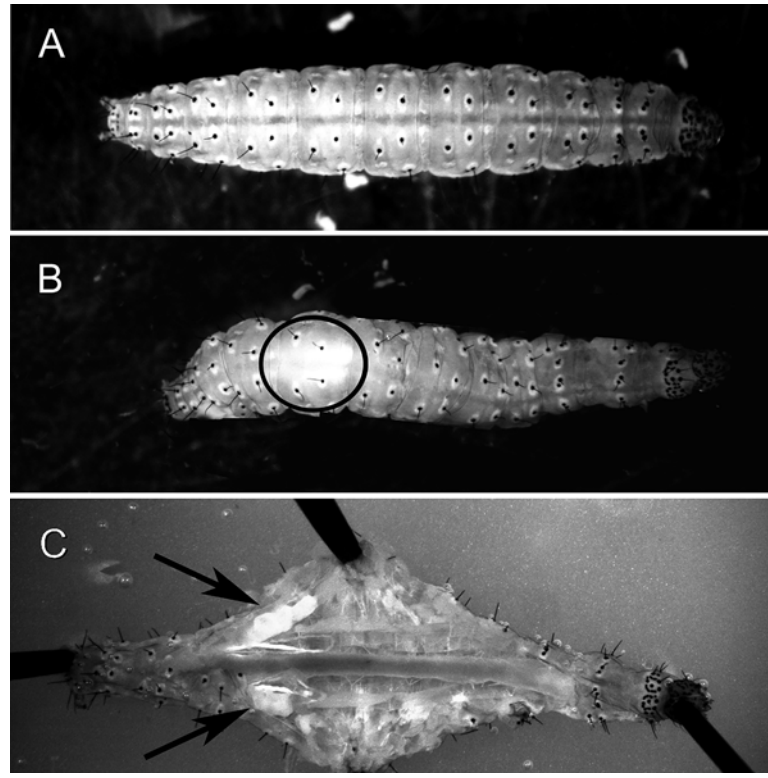


**Fig. 6** *Plutella xylostella* larvae. (**a**) Female last instar. (**b**) Male last instar. The *circle* indicates the location of the testes. (**c**) Dissected male larva, *arrows* indicate testes

### 3.3.9 Imaginal Discs

More challenging than most of the organs/tissues already mentioned are the imaginal discs (Figs. 3 and 4, ID and Fig. 7). These are the tissues in immature insects that are destined to become adult structures. Their name derives from the Latin "imago" in the sense that these structures are the likeness of the adult in the larvae, but they can be so difficult to find that one might think that the term derives from "imaginary." Actually, this is more accurate with Lepidoptera than in some other insects. In *Drosophila* larvae, imaginal discs are loosely connected to the cuticle and can be isolated in mass [2], but the tissues are more tightly connected to the cuticle in lepidopteran larvae. Still, they may be worth the effort to locate because, as undifferentiated cells, they can be effective sources for development of cell lines [3, 4]. Imaginal disc tissues that are destined to become eyes, antennas, legs and wings have been identified, but the wing discs are the easiest to locate and excise in Lepidoptera. Since moths and butterflies have two pairs of wings, they also have two pairs of wing imaginal discs, located in the second (meso-) and third (meta-) thoracic segments, attached to the cuticle near the lateral midline. The discs are nearly transparent,

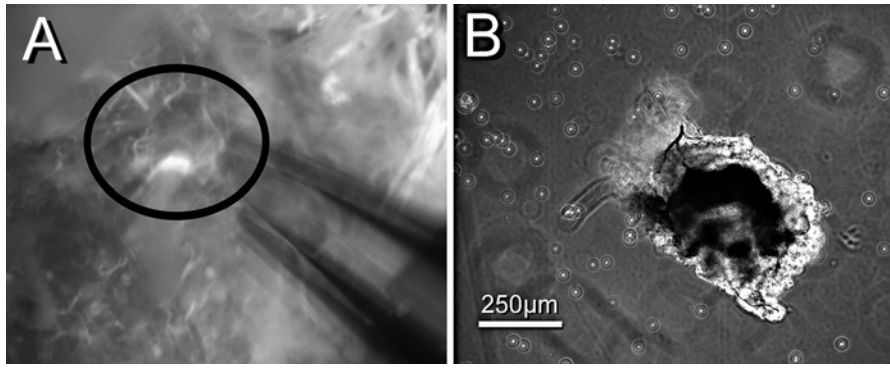


**Fig. 7** Wing imaginal discs of *M. sexta.* (**a**) Disc (*circled*) being slightly lifted from the cuticle with forceps. The *white spot* is the tracheoles. (**b**) Wing imaginal disc in culture. The tracheoles, which are *white* in the other micrographs with reflective light, appear as a dark mass under the transmitted light

but they are each supplied with a large number of tracheoles that are necessary to supply oxygen during the rapid cell growth that occurs late in the last larval instar and pupal stages. As noted earlier, tracheoles are generally highly visible due to the refraction of the air they contain and these can be used to help locate the discs (Figs. 4 and 7).

*3.3.10 Embryos*

Approximately half of the cell lines listed in Chapter 6 were isolated from embryos. Two reasons account for the use of embryos as a favored tissue: (1) embryos are relatively easy to obtain, especially if the insect of interest is being maintained in a laboratory-reared colony and (2) many cells in embryos are actively going through mitosis, increasing the likelihood that they will continue growing in the cultures. The downside to the use of embryos is also related to the fact that many cells are primed to multiply such that the resulting cell cultures may consist of a variety of cell types. In my experience, this has not been an issue with respect to developing cells for baculovirus research since many cells in the insect are capable of replicating the viruses. Techniques described in Subheading 3.8 can be used to isolate different cell types from cultures. Batch methods can be used to initiate cultures from embryos such as the method described by Petersen and Riggs with *Drosophila* [6], but I prefer a more refined approach in which embryos (at a mid-stage of development) are individually isolated from the egg chorion while the eggs are submerged in approximately 1.0 mL of culture medium. If done carefully, then whole embryos will be obtained as shown in Fig. 8a, but more commonly the embryos will become fragmented during removal from the eggs. Twenty to fifty whole and fragmented embryos are transferred to a standing drop (100–150 µL) of culture medium in a 35-mm tissue culture dish (*see* **Note 12**) and cut into smaller pieces with a micro scalpel, resulting in cultures as

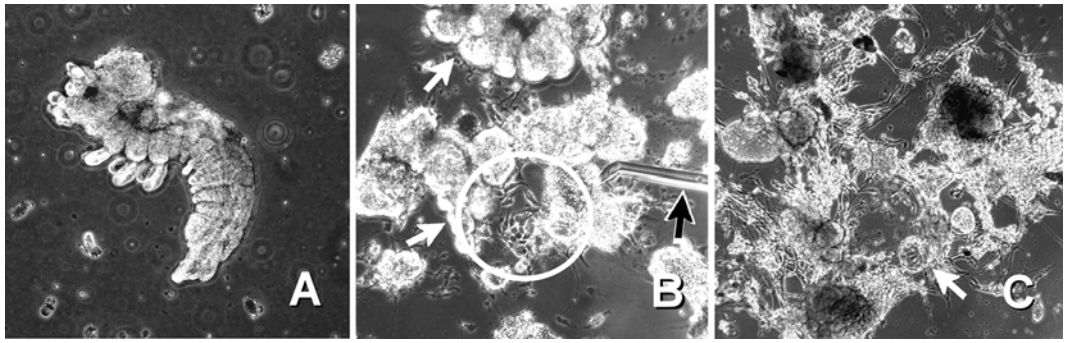


**Fig. 8** Primary embryo culture. (**a**) An intact *Plutella xylostella* embryo after being removed from the chorion. (**b**) Embryo tissues after being cut into pieces with a micro scalpel and incubated for 2 days. The *black arrow* points to a scratch on the Petri dish surface created when the tissues were cut with the micro scalpel; the *white arrows* indicate larger pieces of the embryos; and the *circle* surrounds a small colony of cells attached to the dish which have migrated from the cut tissue fragments. (**c**) A culture after incubation for 10 days. In addition to having more cells that have migrated from the tissues, some vesicles have formed (*arrow*) which consist of epithelial-type cells and often occur in primary embryo cultures. I consider these structures as a positive sign of a healthy culture

seen in Fig. 8b, c. Follow the directions from Subheading 3.4 (**step 6**) for subsequent handling of the culture. The technique for isolating embryo cultures has been described in more detail in an earlier publication [7].

### *3.4 Initiation of Primary Cultures*

1. When the tissue of choice has been identified, it should be carefully excised from the larva and transferred to a 35 mm tissue culture Petri dish containing 1.0 mL culture medium.
2. Hold the tissue in this dish while additional larvae are dissected. Depending on the size of the insect and specific tissue of interest, extracts from several individuals may be necessary. The same dissecting dish can be used for subsequent larvae with the understanding that this also increases the possibility of contaminating the primary culture. A better course of action is to use a separate dish for each dissection, pooling the tissues after completing all the extractions (*see* **Note 13**).
3. Once the tissue extractions are complete, transfer them to a new culture dish containing a standing drop (100–150 μL) of medium or enzyme buffer. At this point, the tissues will each have a distinctive appearance as seen in Figs. 9 and 10.
4. Use a microscalpel to cut the tissue into small fragments, or enzymatically disassociate the tissues with trypsin or another enzyme (*see* **Note 14**).
5. As an alternative to trypsinizing the tissues immediately after dissection, the wounding method can be used and if cells do not begin migrating from the cut tissues, then enzymatic dissociation can be used a few days later.

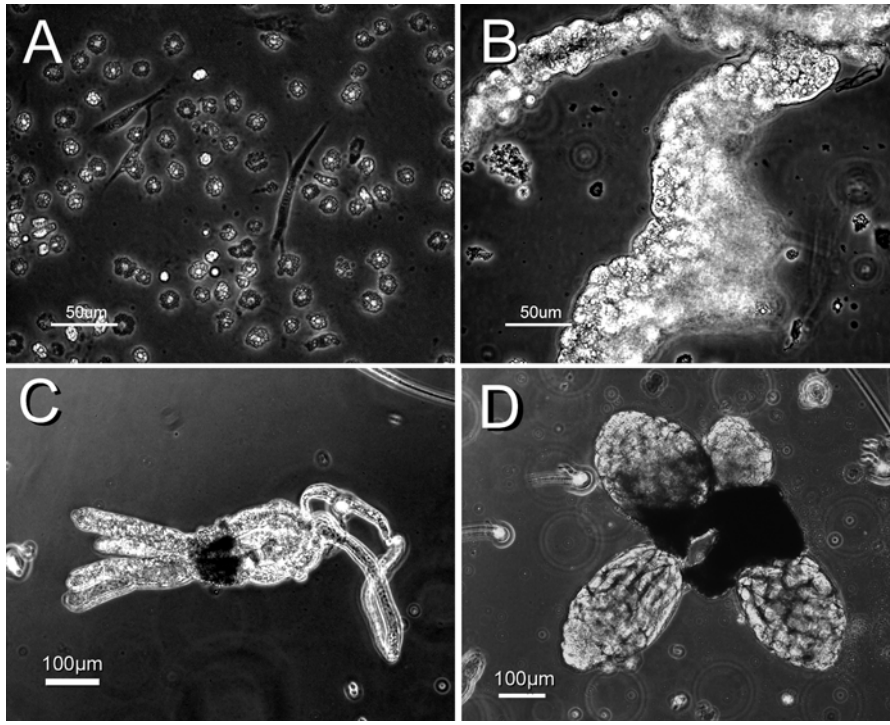


**Fig. 9** Larval cells and tissues in primary cultures. Each of these tissues has been used for establishing cell lines. (**a**) Hemocytes in a dissecting dish (*see* **Note 14**). (**b**) Fat body. (**c**) Ovaries. (**d**) Testis (the sheath has been ruptured showing four follicular bundles)

6. After completing the manipulations, the dish should be sealed by stretching a thin strip of Parafilm® (~8 × 100 mm) around its edge and then placed with other cultures into a small tightly sealed box kept humidified by a small beaker of water or a dampened paper towel.
7. Place the box into a 26–28 °C incubator.

### *3.5 Initial Maintenance*

1. One to two days after initiation, the primary cultures should be examined with an inverted microscope. Any contaminated cultures should be autoclaved and discarded and an additional 1.0 mL medium should be added to all remaining cultures.
2. Reseal the dish with a new piece of Parafilm, replace it into the humidified box, and return it to the incubator.
3. Examine the cultures with the inverted microscope at 7–10 days intervals, adding 0.5 mL fresh medium to all healthy cultures. This routine should be continued until the dish contains sufficient cells for subcultivation.
4. If the volume of medium reaches ~3.5 mL before there are sufficient cells, all but about 0.5 mL should be transferred to a sterile centrifuge tube, the cells pelleted at 50 × *g* for 5 min and the pellet resuspended into 0.5 mL fresh medium and

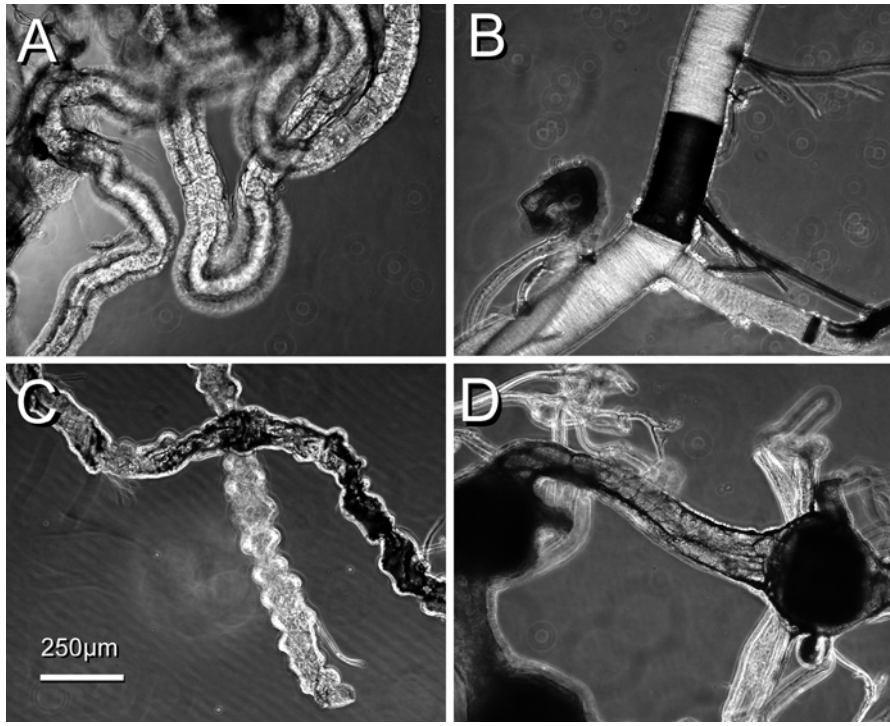


**Fig. 10** Additional larval tissues in primary cultures. The tissues shown in this figure have not been used for establishing cell lines, although some have been used in primary tissue and cell culture studies. (**a**) Salivary gland. (**b**) Tracheoles. (**c**) Malphigian tubules. (**d**) Ventral nerve

returned to the culture dish. The supernatant from this centrifugation can be transferred to a small tissue culture flask and incubated with the primary culture. It is not uncommon to have the low number of cells that are not pelleted at the low speed begin to grow since the medium is somewhat conditioned by the larger tissues. These "pour off cultures" can result in cell strains that are morphologically and functionally distinct from the main cultures. If these cultures appear to contain a significant number of healthy cells, then they should be regularly observed and some of the medium replaced as with the primary cultures.

### *3.6 First Subcultivation*

1. When the primary (or pour off) cultures contain a substantial number of cells (*see* **Note 15**), they can be split into a new culture dish or flask. I prefer to use a small flask (some manufacturers make a 12.5 $cm^2$ version) for the first daughter cultures since these can be tightly capped to reduce the chance of contamination and dehydration.
2. Typically, a gentle rinse of the culture surface can be used for performing this first subculture and the entire medium is transferred from the culture into the new container; 1.0 mL fresh medium is added back to the original dish.

### 3.7 Additional Subcultivations

If there are a substantial number of attached cells remaining, then another subculture may be performed within a week or two. For the second split, a more vigorous method is typically used for detaching the cells.

#### 3.7.1 Cultures in a Flask

1. Chill the culture for 20 min at 4 °C.
2. Use a pipet to flush the growth surface with medium.
3. Transfer contents to a new culture flask with a volume of fresh culture medium equivalent to 50–100 % of the medium from the primary culture.
4. Add fresh medium back to the original culture.

#### 3.7.2 Cultures in a Petri Dish

1. Remove the medium by pipet (this can be added to the "pour off" culture or to the suspended strain created with the first split).
2. Add 1.0 mL of the enzyme buffer to rinse the culture surface.
3. Discard the rinse (or again pool it with the suspended cell culture).
4. Add 0.5 mL trypsin. Different cell strains can respond differently to trypsin, so it is recommended that the culture be checked with an inverted scope at 2–3-min intervals until the cells start to detach.
5. When cells become detached, add 1.0 mL fresh medium and suspend cells by gently drawing in and releasing the medium from the pipet.
6. Transfer the contents to a 12.5 or 25 $cm^2$ flask and rinse the primary dish culture with another 1.0 mL medium, also adding it to the daughter culture. The final volume should be ~2.0 mL in a 12.5 $cm^2$ or 4.0 mL in a 25 $cm^2$ flask.
7. Add 1.0 mL fresh medium back to the original primary culture and reseal with Parafilm®.

These various subculturing methods typically lead to different cell strains that will have different properties such as susceptibilities to or productivities of viruses. Even if these features are not different, the cultures can be used for different purposes (plaque assaying/cloning with the attached strain, large scale suspension cultures with the unattached strain).

### 3.8 Strain Selection

#### 3.8.1 Growth Rate

As soon as cells begin growing in a primary culture, a Darwinian natural selection process begins based on a number of characteristics. The most obvious of these is growth rates. For example, if two cells exist in a culture with the cell cycle of one of them occurring in half the time of the other, then the slower cell type will be outnumbered a thousand to one within ten of its divisions and a million to one within 20. Every time you split such a culture, the proportion of faster growing cells becomes greater until none of the slow growing cells are transferred to the new culture.

### 3.8.2 Attachment

Other features besides growth rates can also influence the distribution of cell types in the culture. For example, suppose in the previous example the faster growing cells were much more adherent to the culture flask such that every time you split cells using the flushing method (described in Chapter 9), half of them were either mortally damaged or remained attached to the old culture. In this situation, if you split the cultures at a 1:2 ratio at each passage, the fast- and slow-growing cells would exist at an equal level in each new culture. Alternatively, if the normal trypsin method was used for subculturing (also described in Chapter 9), then loosely and non-attached cells are removed from the culture prior to the enzyme treatment and thus those types of cells would be depleted from the cell line very quickly. Because of these and other factors affecting the cell population in cultures, more than one subculture method is often used, especially on early passage cultures. This approach will ensure that the widest diversity of cell types is maintained until it becomes apparent which cell type is most useful for a given application. As an alternative to varied subculture methods, cells could be cloned at an early passage.

## 4 Notes

1. Several manufacturers now supply insect cell culture media and many of these are specifically designed for lepidopteran cultures. Previously, my medium of choice was TC-100 (originally described as BML-TC/10 [5]) with some additional supplements (peptones, additional vitamins, and trace minerals as described previously [7]), but in recent efforts, both I [8] and other researchers [9] have used the serum-free formulations such as the Ex-Cell 400 series of media (JRH Biologicals, Lenexa, KS), Sf-900II (Invitrogen/GIBCO, Carlsbad, CA), or Insect Express (HyClone, Logan, UT). These can support growth of established insect cell lines without any additional supplements, but the addition of a small quantity, typically 5–10 % (v/v) of fetal bovine serum (FBS) is recommended when using them in attempts to establish new lines. FBS is known to provide growth factors, typically small proteins that stimulate cell division in vertebrate cells. Little is known about these factors in insects but homologs to the mammalian genes for these factors have been found in some insects. Still, even if the mammalian factors are different, some positive effects are observed with FBS in insect cell cultures making it an effective supplement for primary culture work.
2. In Chapter 9 I expound on the virtues of not using antibiotics in continuous cell lines. Unfortunately, it is a fantasy to expect primary cultures from insect tissue to remain uncontaminated without the use of an antibiotic. Gentamycin is a

broad-spectrum antibiotic that has relatively mild or no toxicity to eukaryotic cells. Researchers also have successfully used a penicillin-streptomycin combination (typically used at 100 units penicillin and 0.1 mg/mL streptomycin culture medium) to reduce contamination in primary insect cultures. Antifungal agents are particularly toxic to insect cells [10], probably because the biochemical pathways they target also occur in insects and thus I do not recommend their use in efforts to establish new cell lines.

3. Most researchers working with cell cultures and viruses will find a biological safety cabinet a better choice for routine culture work. Unfortunately, most of these have a glass front that makes it impossible to use a microscope in the hood. A clean bench is much more appropriate for performing dissections while using a microscope but, unless you are performing a lot of dissections, it may not be worth the extra expense of having both types of hood. If your facility has many other researchers, you may want to see if you can use someone else's hood for these procedures. Alternatively, the dissection can be performed without a hood. In this case, you should select a small room that can be kept closed during the dissection to minimize airflow across the field.
4. These are available from suppliers specializing in surgical equipment since they are also used in procedures such as eye surgery and other delicate operations.
5. Placing a layer of cotton in the bottom of the jar serves two purposes. First, the delicate tools can be placed in the alcohol with less chance of damaging the tips and, second, the cotton can be used to wipe off tissue fragments that adhere to the instruments during the dissections.
6. The wind screen is helpful in keeping the flame steady while working in the laminar flow hood's air current. I have made one from a one-pound coffee can (Fig. 1). A similar structure can be constructed from a heavy gauge aluminum foil.
7. Most lepidopteran larvae live in relatively clean environments. If you are using a laboratory colony on artificial diet, then you may want to consider adding some antibiotics to the diet for the insects you use for setting up cultures. If initial efforts with a short disinfection time results in many contaminated cultures, then a longer disinfection with 70 % ethanol can be used or 0.05 % $HgCl_2$ in 70 % ethanol can be used instead. If the tissues of interest are embryos, then the eggs can be pretreated for 1–2 min in 2.6 % sodium hypochlorite (=50 % (v/v) household bleach in distilled water), which will soften the chorion to make removal of the embryos easier. The eggs are then rinsed at least three times with sterile distilled water prior to disinfection with ethanol (5 min should be sufficient since the sodium hypochlorite is also a disinfecting solution).

8. Care must be taken in flaming the instruments. Hold the instrument nearly parallel to the floor, with the tips just slightly lowered. Having the tips higher than your hand can result in the burning alcohol flowing down the instrument onto your hand, while holding them with the tips directly below your hand will result in the heat of the flame being directly below your fingers. Since the laminar flow clean bench is also blowing air toward you, the flaming instruments should be held on an angle so that your hand is not directly behind the flame.
9. Pinning the insect in this way serves a couple of functions. Most importantly, it immobilizes the larva so that the dissection can be performed more easily. In addition, many lepidopteran larvae regurgitate or defecate when they are disturbed. Although the anesthetic effect of the submersion in ethanol and water rinses should minimize this, inserting the pins as I have described will effectively block these activities. Of course, the digestive tract is almost certainly pierced when you do this, so the pins should not be removed until the dissection is complete and a new dish must be used for the next larva or there will be a greater risk of contamination from the gut contents.
10. If you frequently rupture the digestive tract during the dissection, then I suggest starving the insect for a few hours to reduce the amount of gut contents.
11. These have never been used for initiating cell lines from Lepidoptera but the results by Engelhard et al. [11] indicate they could be extremely useful as indicator cells since in some virus/insect systems, they are responsible for initially spreading the infection.
12. Moving embryos from the initial dish to the standing drop of medium can be challenging since the tissue is very fragile. Researchers with a lot of experience in manipulating small fragments will be able to use jeweler's forceps, but an alternative method is to use sterile Rainin-style pipet tips to collect the tissue fragments and transfer them. If this method is used, then care should be used to transfer a minimum amount of the medium from the original dish.
13. While I suggested using a wax dish and insect pins in the previous section to aid in the dissection, as you become accustomed to manipulating larvae and the dissecting instruments, you may be able to leave the insect unpinned and simply grasp it with a pair of forceps while cutting the cuticle and isolating the tissue. An advantage of this technique is that a tissue culture-grade dish can be used in the initial dissection and, after the tissues of interest are removed, this dish will contain a substantial number of hemocytes and can become a primary culture itself (Fig. 9a).

14. Many insect tissues have a wound response to cuts and will initiate cell division spontaneously. However, treatment with a mild concentration of trypsin, i.e., a concentration that is insufficient to completely disassociate the tissue into single cells, may improve migration and growth of cells from the tissue explants. Specifically, the tissue is treated for 5–20 min with 50 μg/mL trypsin in divalent-free PBS. Following treatment, the trypsin solution is replaced with medium (100–150 μL in a standing drop) containing 50 μg/mL gentamicin. Two forceps are then used to tear the tissue into smaller fragments.
15. The cell number necessary for successful subcultivation is not a precise quantity and is one of the decisions that become easier as you gain experience. If the cells become too dense, then there is a chance that they will deplete the nutrients in the medium and growth will stop. Alternatively, many cells produce autocrine growth factors that stimulate their own growth (as well as that of neighboring cells). If cultures are split too early to result in very low cell densities, then these growth factors will disperse into the medium, thereby causing a reduced or even inhibited cell growth.

## Acknowledgements

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# Chapter 8

# Development of Serum-Free Media for Lepidopteran Insect Cell Lines

**Leslie C.L. Chan and Steven Reid**

## Abstract

Baculovirus-based Insect Cell Technology (ICT) is widely used for the expression of recombinant heterologous proteins and baculovirus bioinsecticides, and has recently gained momentum as a commercial manufacturing platform for human and veterinary vaccines. The three key components of ICT are the Lepidopteran insect cell line, the baculovirus vector, and the growth medium. Insect cell growth media have evolved significantly in the past five decades, from basal media supplemented with hemolymph or animal serum, to highly optimized serum-free media and feeds (SFM and SFF) capable of supporting very high cell densities and recombinant protein yields. The substitution of animal sera with protein hydrolysates in SFM results in greatly reduced medium costs and much improved process scalability. However, both sera and hydrolysates share the disadvantage of lot-to-lot variability, which is detrimental to process reproducibility. Hence, the industrialization of ICT would benefit greatly from chemically defined media (CDM) for insect cells, which are not yet commercially available. On the other hand, applications such as baculovirus bioinsecticides would need truly low cost serum-free media and feeds (LC-SFM and LC-SFF) for economic viability, which require the substitution of a majority of expensive added amino acids with even higher levels of hydrolysates, hence increasing the risk of a variable process. CDM developments are anticipated to benefit both conventional and low cost ICT applications, by identifying key growth factors in hydrolysates for more targeted media and feed design.

**Key words** Insect cell technology, Baculovirus expression vector system, Serum-free medium, Chemically defined medium, Protein hydrolysates, Lepidopteran cell lines

## 1 Introduction

Insect Cell Technology (ICT), incorporating the Baculovirus Expression Vector System (BEVS), is widely used for the expression of recombinant heterologous proteins in research laboratories due to its ability to express near-authentic eukaryotic proteins quickly and in large quantities [1, 2]. In recent years, improvements to and new applications for ICT have emerged, including transgenic insect cell lines capable of humanized protein glycosylation, virus-like particle (VLP) vaccines, gene delivery vectors for mammalian cells, and using baculovirus to display peptides or

David W. Murhammer (ed.), *Baculovirus and Insect Cell Expression Protocols*, Methods in Molecular Biology, vol. 1350, DOI 10.1007/978-1-4939-3043-2_8, 

proteins as immunogens [1]. ICT has also become increasingly important as a manufacturing platform for human and veterinary vaccines, as shown by the recent commercialization of Cervarix® (cervical cancer), Provenge® (prostate cancer), and Ingelvac® Circoflex™ (porcine circovirus) [3]. ICT mainly involves the use of Lepidopteran insect cell lines, e.g., Sf-9 (*Spodoptera frugiperda*) or BTI-Tn-5B1-4 (*Trichoplusia ni*), to propagate the dominant baculovirus expression vector, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). ICT is also used to propagate wild-type baculoviruses for use as bioinsecticides in Lepidopteran cell lines such as Sf-9, HzAM1 (*Helicoverpa zea*), saUFL-AG-286 (*Anticarsia gemmatalis*), and Ld652Y (*Lymantria dispar*) [4–7].

The majority of commercially sourced growth media are designed for supporting the growth of and the production of AcMNPV-expressed recombinant proteins in Sf-9 or BTI-Tn-5B1-4 (Tn-5) cells. The commercial name of Tn-5 cells is High-Five™ (Life Technologies). Informative reviews on the development and formulation of insect cell media have been presented previously, including one that focuses mainly on Lepidopteran insect cell cultures [8] and another that describes a wider variety of media formulations for insect cell lines derived from Lepidopteran, Dipteran, and Hemipteran orders [9]. The development of growth media for both insect and mammalian cell cultures has strong parallels. For example, the basal medium is generally formulated to mimic the chemical composition and physico-chemical parameters of the animal's circulatory fluid, i.e., blood plasma for mammals [10] and hemolymph for insects [11]. Furthermore, trends in mammalian medium development have been adopted by insect media formulators, such as the use of vertebrate sera [12, 13] and protein hydrolysates [14] for growth promotion, serum-free media (SFM) [13], and the use of Pluronic F-68 (a surfactant) to protect fragile animal cells (no cell walls) from shear stresses and bubble damage in bioreactor suspension cultures [15, 16].

A timeline of the development of media for Lepidopteran insect cell cultures is presented in Table 1. The earliest basal media include Wyatt's [11] and Grace's [17], which were followed by more refined basal formulations such as TNMFH [18], TC100 [19], and IPL41 [20]. Insect cell basal media are chemically defined (containing amino acids, organic acids, sugars, inorganic salts, and vitamins), but are generally unable to support insect cell proliferation unless they are supplemented with undefined components such as heat-treated hemolymph or vertebrate serum. Serum is usually added at 5–10 (v/v)% in serum-containing media (SCM) [8], and is a more practical supplement than hemolymph because it is readily available at larger volumes. Serum (blood plasma with clotting factors removed) is a complex mixture of all major nutritional classes, including proteins, peptides, amino acids, organic acids, carbohydrates, fatty acids, sterols, vitamins, and

**Table 1**
**Lepidopteran insect cell culture media at different developmental periods, demonstrating the transition from serum-dependent to serum-free formulations**

| Complete medium | Year published or available | Basal medium (Chemically defined) | Supplements | Lepidopteran cells supported (Examples) | References |
|---|---|---|---|---|---|
| *Hemolymph/Serum Containing Media (HCM/SCM)* | | | | | |
| Wyatt's | 1956 | Wyatt's | Hemolymph | Bm | [11] |
| Grace's | 1962 | Modified Wyatt's | Hemolymph, Albumin | Ae | [17] |
| MM | 1964 | Salts, glucose only | FBS, YST, LAC | Mb | [85, 86] |
| TNMFH | 1970 | Grace's | FBS, YST, LAC | Tn | [18] |
| TC100 | 1975 | Modified Grace's | FBS, TB | Tn, Sf, Hz | [19] |
| IPL40, IPL45 | 1975 | Modified Grace's | Multiple vertebrate sera, YST, LAC | Tn, Sf, Hz | [87] |
| IPL41 | 1981 | Modified IPL45 | FBS, TPB | Sf | [20] |
| *Serum Free Media (SFM)* | | | | | |
| MM-SF | 1982 | MM | YST, LAC | Mb | [86] |
| ISFM | 1988 | IPL41 | LipidEm, YST | Sf | [36, 37] |
| Ex-Cell 400 series | Since 1990s | IPL41 | LipidEm, YST | Sf | MM |
| Sf-900 series | Since 1990s | IPL41 | LipidEm, YST | Sf, Hz | MM [88] |
| Express Five | Since 1990s | IPL41 | LipidEm, YST | Tn | MM |
| *Chemically Defined Media (CDM)* | | | | | |
| L21 CDM | 1976 | – | No lipids added in CDM | Md | [53, 89] |
| Wilkie's CDM | 1980 | – | Sterols & fatty acids included in CDM | Sf | [54, 89] |
| MTCM-1520 CDM | 1996 | – | Sterols & fatty acids included in CDM | Dipteran cells only | [55] |
| *Low Cost Serum Free Media (LC-SFM)* | | | | | |
| SF1 | 1996 | Modified IPL41 | LipidEm, YST, PRI, LAC | Tn, Sf | [8] |
| ISYL | 1998 | IPL41 | LipidEm, YST, SOY | Tn | [40] |
| YPR | 2001 | IPL41 | LipidEm, YST, PRI | Tn, Sf | [32] |

(continued)

**Table 1 (continued)**

| Complete medium | Year published or available | Basal medium (Chemically defined) | Supplements | Lepidopteran cells supported (Examples) | References |
|---|---|---|---|---|---|
| LCM | 2003 | Modified IPL41 | LipidEm, YST, PRI, LAC, CAS | Hz | [90] |
| VPM3 | 2005 | Modified IPL41 | LipidEm, YST, PRI, LAC, SOY, CAS | Hz | [41] |
| UNL10 | 2009 | In-house design | LipidEm, YST, CAS, TPB | Ag | [42] |
| VPM3 2-HYD | 2012 | Modified IPL41 | LipidEm, YST-AMP or YST-POT | Hz | [34] |

*Acronyms/Abbreviations*: *AMP* amyl meat peptone, *CAS* casein hydrolysate, *FBS* fetal bovine serum, *LAC* lactalbumin hydrolysate, *LipidEm* lipid emulsion, manufactured, containing cholesterol, fatty acids, Pluronic F-68, *MM* manufacturer's manual, *POT* potato hydrolysate, *PRI* primatone RL, *SOY* soy hydrolysate, *TB* tryptose broth, *TPB* tryptose phosphate broth, *YST* yeast extract

*Insect cell lines*: Ae (*Antheraea eucalypti*), Ag (*Anticarsia gemmatalis*), Bm (*Bombyx mori*), Hz (*Helicoverpa zea*), Mb (*Mamestra brassicae*), Md (*Malacosoma disstria*), Sf (*Spodoptera frugiperda*), Tn (*Trichoplusia ni*)

inorganic salts/trace elements [21, 22]. It confers multiple growth promotion functionalities, including cell adhesion (for stationary cultures), shear protection (for suspension cultures), iron transport/delivery (e.g., transferrin), detoxification (e.g., selenium), anti-oxidation (e.g. glutathione), anti-proteolysis, and growth stimulation (e.g., hormones) [8, 23–27].

### 1.1 Serum-Free Media

There are major disadvantages in using vertebrate serum, which are due to its costliness, protein content (which interferes with downstream purification processes), potential harboring of adventitious particles (e.g., viruses, prions, and mycoplasma), and lot-to-lot variability affecting process reproducibility [8, 22, 23, 25]. Serum may also exhibit toxicity (e.g., from bilirubin) [22], that affects certain insect cell lines (e.g., *T. ni* cells) [28], and have been shown to depress baculovirus production in *H. zea* cells [5]. Hence, it is advantageous to remove serum from insect media formulations, especially for suspension culture applications requiring bioreactor scale-up. Protein hydrolysates represent an efficacious substitute for serum since they also are good sources of oligopeptides, amino acids, carbohydrates, vitamins, trace elements, undefined growth factor analogues, and metal-chelating capacity [8, 25, 29, 30], while being much cheaper, protein-free, and available at large quantities for scale-up [25]. Protein hydrolysates may contain between 1–50 % free amino acids and 60–80 %

total amino acids (free and oligopeptide form) [8, 25]. Most protein hydrolysates (or peptones) are enzymatic digests of crude animal or plant proteins [25, 31]. For convenience, yeast extract is considered a protein hydrolysate in this chapter, although it is technically an autolysate of yeast cells. Yeast extract is a particularly efficacious growth promoter for insect cell cultures, as shown by a factorial screening experiment comparing the performance of a wide selection of protein hydrolysates from microbial, animal, and plant origins [32]. In general, there is a positive correlation between yeast extract concentration and the peak cell density (PCD) obtained from uninfected cultures, although there is an upper concentration limit due to cell toxicity [32, 33]. Primatone RL (meat peptone) is another commonly used hydrolysate, but its efficacy lies in prolonging cell viability (anti-apoptotic effect) [30] rather than in cell growth promotion [32, 34].

Hydrolysates have been used in tandem with serum in earlier Lepidopteran media formulations (e.g., MM, TNMFH, TC100 and IPL series), but hydrolysate-containing serum-free media were developed subsequently, such as MM-SF medium (Table 1). However, hydrolysates cannot adequately replace the sterol content and shear-protection attributes of sera, necessary for optimal baculovirus infection and suspension cultivation, respectively [35]. Hence, a breakthrough in Lepidopteran SFM formulation was the development of a manufactured lipid emulsion to supply these functionalities: containing cholesterol, an undefined source of fatty acids (cod liver oil methyl esters), and the shear-protectant Pluronic F-68 [36, 37]. Yeast extract and the lipid emulsion were added to IPL41 basal medium to form ISFM, which was the first Lepidopteran SFM shown to be efficacious for supporting insect cell growth and the production of ICT-expressed recombinant proteins in gas-sparged suspension-based bioreactors [36, 37]. IPL41-based SFM then informed the development of refined commercial derivatives such as the Ex-Cell™ 400 (Sigma-Aldrich) and Sf-900™ (Life Technologies) SFM series for Sf-9 cells, and Express Five® SFM (LT) for Tn-5 cells [8] (Table 1). A selection of currently available commercial Lepidopteran SFM, suitable for suspension cell cultures, and the cell lines that each medium supports, is presented in Table 2. Furthermore, a subset of these commercial SFM is presented in Table 3, showing each medium's efficacy (where information is available) in terms of the support of uninfected PCD, and the maximum volumetric yields of budded virus (BV) and recombinant protein/occlusion body (OB) in baculovirus-infected cells, and the infected PCD range at which this occurs. Many of these commercial insect cell SFM are described as protein-free formulations, although animal cell SFM do not necessarily have to be protein-free. For example, purified proteins such as bovine serum albumin (BSA) may be included in SFM formulations for specific functionalities, e.g., as a carrier of

**Table 2**
**Commercially available serum-free media (SFM) for suspension cultures of Lepidopteran insect cells (with proprietary formulations)**

| | Manufacturer | Medium | ACF | RHF | Gln | Cell-lines supported (examples from MM) |
|---|---|---|---|---|---|---|
| 1 | Allele Biotech. | SFICM (Sapphire™) | No | No | Yes | Sf9, Sf21, Tn5 |
| 2 | Applichem | AC Insect | No | No | ND | Sf9, Tn5 |
| 3 | BD Biosciences | Max-XP (BD BaculoGold™) | No | No | ND | Sf9, Sf21 |
| 4 | Biochrom AG | Insectomed SF Express | No | No | Yes | Sf9, Sf21, Tn5 |
| 5 | Biological Industries | BIOINSECT-1 | No | No | Yes | Sf9, Tn5 |
| 6 | Corning | Insectagro Sf9™ | No | No | Yes | Sf9, Sf21 |
| 7 | Cosmo Bio | COSMEDIUM 009 | No | No | ND | Sf9 |
| 8 | Expression Systems | ESF 921 | No | No | ND | Sf9, Sf21, Tn5 |
| 9 | Expression Systems | ESF AF | Yes | No | ND | Sf9, Sf21, Tn5 |
| 10 | Irvine Scientific | IS BAC™ | No | No | ND | Sf9, Sf21, Tn5 |
| 11 | Kohjin Bio | KBM710 | No | No | ND | Sf9 |
| 12 | Life Technologies | Sf-900™ II | No | No | Yes | Sf9, Sf21, Ld, Tn368, |
| 13 | Life Technologies | Sf-900™ III | Yes | Yes | Yes | Sf9, Sf21 |
| 14 | Life Technologies | Express Five® | No | No | AS | Tn5 |
| 15 | Lonza | Insect-XPRESS™ | No | No | Yes | Sf9, Sf21 |
| 16 | Merck Millipore | Tri-Ex™ | No | No | ND | Sf9 |
| 17 | MP Biomedicals | SFPFIM | No | No | Yes | Sf9, Tn5 |
| 18 | Oxford Expression Tech. | baculoGROW™ | No | No | ND | Sf9, Sf21, Tn5 |
| 19 | Sigma-Aldrich | EX-CELL™ 420 | No | No | Yes | Sf9, Sf21 |
| 20 | Sigma-Aldrich | EX-CELL™ 405 | No | No | Yes | Tn5 |
| 21 | Sigma-Aldrich | TiterHigh™ Sf | Yes | No | Ala-Gln | Sf9, Sf21 |
| 22 | Thermo Scientific | SFM4-Insect™ (HyClone®) | Yes | No | Yes | Sf9, Sf21, Tn5 |
| 23 | Thermo Scientific | SFX-Insect™ (HyClone®) | No | No | ND | Sf9, Sf21, Tn5 |

*Acronyms/Abbreviations*: *ACF* animal component free, *AS* added separately, *MM* manufacturer's manual, *ND* not disclosed, *RHF* reduced hydrolysate formulation, *SFICM* serum-free insect culture medium, *SFPFIM* serum-free protein-free insect medium

*Insect cell lines*: HzAM1 (*Helicoverpa zea*), Ld (*Lymantria dispar*), Sf9 (*Spodoptera frugiperda*), Sf21 (*S. frugiperda*), Tn5 (*Trichoplusia ni*), Tn368 (*T. ni*)

**Table 3**
**Performance of a selection of commercial and low-cost serum-free media (SFM), when used to support Lepidopteran insect cell growth and baculovirus infection in batch suspension cultures**

| | Medium | Cell line | Baculovirus | Uninfected PCD ($10^6$ cells/mL) | Maximum BV Titre (PCD) ($10^9$ PFU/mL) ($10^6$ cells/mL) | Maximum Protein Yield (PCD) (Various Units) ($10^6$ cells/mL) | References |
|---|---|---|---|---|---|---|---|
| *Commercial SFM* | | | | | | | |
| 1 | BaculoGROW™ | Sf9 | rAcMNPV | >10 | ND | ND | MM |
| 2 | ESF 921, ESF AF | Sf9 | rAcMNPV | >12 | ND | ND | MM |
| 3 | EX-CELL™ 405 | Tn5 | rAcMNPV | 6 | ND | 456 IU/mL β-Gal (1.6–2) | [91] |
| | | Tn5 | rAcMNPV | 3.6 | ND | 2.7 IU/mL SEAP (~1) | [92] |
| | | Tn5 | wtAcMNPV | 3.6 | ND | $1.6\times10^8$ OB/mL (~1) | [92] |
| 4 | EX-CELL™ 420 | Sf9 | rAcMNPV | >10 | ND | ND | MM |
| 5 | Express Five® | Tn5 | rAcMNPV | 5–6 | ND | $1.7\times10^5$ U/$10^6$ cells | MM |
| | | Tn5 | rAcMNPV | 3.1 | ND | 4.2 IU/mL SEAP (~1) | [92] |
| | | Tn5 | wtAcMNPV | 3.1 | ND | $1.6\times10^8$ OB/mL (~1) | [92] |
| 6 | Insect-XPRESS™ | Sf9 | rAcMNPV | >8.3 | ND | ND | MM |
| 7 | Sf-900™ II | Sf9 | rAcMNPV | 9–12 | ND | $5\times10^5$ U/mL β-Gal (~3) | MM |
| | | Sf9 | rAcMNPV | 9 | 12–30 (3–4) | $6–7\times10^5$ U/mL β-Gal (3–4) | [56] |
| | | Sf9 | rAcMNPV | 20 | 5 (8–11) | $8–9\times10^5$ U/mL β-Gal (8–11) | [57] |
| | | Sf9 | rAcMNPV | 9 | ND | 150–200 U/mL β-Gal (3–4) | [64] |
| | | Sf21 | rAcMNPV | 4–5 | ND | 282 IU/mL β-Gal (2.1–3) | [91] |
| | | HzAM1 | wtHearNPV | 4–5 | 0.02–0.03 (0.5–1) | $9–12\times10^7$ OB/mL (1–2) | [5, 63, 90] |
| 8 | Sf-900™ III | Sf9 | rAcMNPV | 10–14 | ND | ND | MM |
| 9 | TiterHigh™ Sf | Sf9 | rAcMNPV | >10 | ND | ND | MM |

(continued)

**Table 3**
**(continued)**

| | Medium | Cell line | Baculovirus | Uninfected PCD ($10^6$ cells/mL) | Maximum BV Titre (PCD) ($10^9$ PFU/mL) ($10^6$ cells/mL) | Maximum Protein Yield (PCD) (Various Units) ($10^6$ cells/mL) | References |
|---|---|---|---|---|---|---|---|
| *Low cost SFM* | | | | | | | |
| 1 | ISFM (IPL41 SFM) | Sf9 | rAcMNPV | 5–6 | 0.2 | 40 mg/L CSF (~3) | [36, 37] |
| 2 | ISYL | Tn5 | rAcMNPV | 5–6 | ND | 50 IU/L SEAP (~4) | [40] |
| 3 | LCM | HzAM1 | wtHearNPV | 5.6 | ND | $5–7 \times 10^7$ OB/mL (1–2) | [90] |
| 4 | SF1 | Sf9 | rAcMNPV | 8–11 | ND | ND | [8] |
| 5 | UNL10 | saUFL-AG-286 | wtAgMNPV | 2–3 | 0.6 $TCID_{50}$/mL (0.8) | $3–3.5 \times 10^8$ OB/mL (0.4–0.8) | [6, 42] |
| 6 | VPM3 | HzAM1 | wtHearNPV | 3–4 | 0.04–0.06 (2) | $4–6 \times 10^8$ OB/mL (1–1.5) | Reid Lab. |
| 7 | YPR | Sf9 | rAcMNPV | 5.4 | ND | 28 IU/mL SEAP (~2.4) | [32] |
| | | Tn5 | rAcMNPV | 6.1 | ND | 43 IU/mL SEAP (~3.5) | [32] |

*Acronyms/Abbreviations*: *BV* budded virus, *MM* manufacturer's manual, *ND* not disclosed, *PCD* peak cell density
*Insect cell lines*: HzAM1 (*Helicoverpa zea*), saUFL-AG-286 (*Anticarsia gemmatalis*), Sf9 (*Spodoptera frugiperda*), Sf21 (*S. frugiperda*), Tn5 (*Trichoplusia ni*)

bioactive small molecules [38]. Some of the SFM in Table 2 are also described as animal component-free (ACF) formulations, which address regulatory concerns on transmissible spongiform encephalopathies (TSE) [39].

### 1.2 Low-Cost Serum-Free Media

Vertebrate serum (e.g., fetal bovine serum, FBS) is an expensive commodity, and its omission in SFM represents a large reduction in raw materials cost. For example, if a cost analysis is performed on all components (cell-culture grade when available) of a medium formulation based on the cheapest available non-bulk retail prices from a supplier such as Sigma-Aldrich, then the cost contribution of FBS (at 10 % (v/v)) to an IPL41-based SCM is approximately 2/3 of the total materials cost (calculations not shown). When FBS is replaced by yeast extract (at 4 g/L) and a lipid emulsion, the resulting SFM is about 1/3 of the SCM cost. The 21 pure amino acids in IPL41 [20], however, are also expensive components and contribute approximately 30 % of the materials cost of an IPL41-based SCM, which increases to more than 80 % in the serum-free version.

Hence, there is much opportunity to reduce medium costs further by reducing the free amino acids content and increasing the proportion of protein hydrolysates (which are significantly cheaper than the former). A low-cost serum-free medium (LC-SFM) is obviously a critical component of a low-cost ICT production process, which contributes to the economic viability of applications such as baculovirus bioinsecticides, animal vaccines, and biosimilars. A significant number of LC-SFM for Lepidopteran insect cell suspension cultures have been described in the literature (Table 1), which are demonstrated to support good uninfected PCDs, and maximum volumetric yields of BV and recombinant protein/OB in baculovirus-infected cells, comparable to or better than those in commercial SFM (Table 3). Most of these LC-SFM are based on the IPL41 basal formulation, and are supplemented with a lipid emulsion and a range of hydrolysates (*see* Table 4 for a detailed list of ingredients in these media). The ISYL [40] and YPR [32] SFM are described as low-cost formulations, but are not truly low cost since they incorporate the complete IPL41 basal medium that contain 21 added amino acids [8, 20]. ISYL (6 g/L yeast extract and 4 g/L soy hydrolysate) and YPR (6 g/L yeast extract and 5 g/L Primatone RL) are reported to support up to $6 \times 10^6$ cells/mL of Tn-5 and Tn-5/Sf-9 cells, respectively.

Truly low-cost SFM have reduced numbers and/or concentrations of added amino acids, and these include the SF1 [8], VPM3 [41] and UNL10 [42] formulations. The original SF1 formulation contains one added amino acid (glutamine) and three hydrolysates (5.6 g/L each of yeast extract, Primatone RL and lactalbumin hydrolysate), and supports PCDs of $5–6 \times 10^6$ cells/mL (Sf-9). However, a fortified version of SF1 was required to reach higher

**Table 4**
**A selection of low cost serum-free media (LC-SFM) for suspension cultures of Lepidopteran insect cells, with reported formulations (the number of chemicals in each nutrient group are indicated)**

| Nutrient groups | ISFM (IPL41 SFM) | ISYL | YPR | SF1[a] | VPM3[b] | UNL10 |
|---|---|---|---|---|---|---|
| Amino Acids | 21<br>Ala, Arg, Asn, Asp, Cys-Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Hyp, Ser, Thr, Trp, Tyr, Val | 21<br>Same as ISFM | 21<br>Same as ISFM | 1<br>Gln, same level as ISFM | 5<br>Asn, Cys-Cys, Glu, Lys, Met, at different conc. to ISFM | 20<br>Same as ISFM, except no Hyp, & at reduced conc. generally |
| Organic Acids | 4<br>Fumaric, Ketoglutaric, Malic, Succinic Acids | 4<br>Same as ISFM | 4<br>Same as ISFM | 4<br>Same as ISFM[c] | 0 | 3<br>Fumaric, Ketoglutaric, Malic Acids |
| Inorganic Salts (Trace Elements) | 12<br>$CaCl_2$, KCl, $MgSO_4$, NaCl, $NaHCO_3$, $NaH_2PO_4$ (Co, Cu, Fe, Mn, Mo, Zn) | 12<br>Same as ISFM | 12<br>Same as ISFM | 12<br>Same as ISFM[c] | 12<br>Same as ISFM, at similar conc. | 11<br>Similar to ISFM, except at different conc. generally |
| Vitamins | 11<br>Biotin, B Vitamins ($B_1$, $B_2$, $B_3$, $B_5$, $B_6$, $B_{12}$), Choline Chloride, Folic Acid, myo-Inositol, PABA | 11<br>Same as ISFM | 11<br>Same as ISFM | 0 | 12<br>Same as ISFM, & Inosine additionally | 11<br>Same as ISFM, but at different conc. generally |
| Sugars | 3<br>Glucose, Maltose, Sucrose | 3<br>Same as ISFM | 3<br>Same as ISFM | 1<br>Glucose | 5<br>Galactose, Glucose, Maltose, Sucrose Trehalose | 1<br>Glucose |
| Lipid Emulsion | 6<br>Cholesterol, Cod Liver Oil, Ethanol, Pluronic F-68, Tween 80, Vitamin E | 6<br>Same as ISFM | 6<br>Same as ISFM | 6<br>Same as ISFM | 7<br>Same as ISFM but at slightly different conc., & Soy Lecithin additionally | 7<br>Same as ISFM, but Cod Liver Oil is replaced by Soybean Oil |

(continued)

**Table 4 (continued)**

| Nutrient groups | ISFM (IPL41 SFM) | ISYL | YPR | SF1[a] | VPM3[b] | UNL10 |
|---|---|---|---|---|---|---|
| Protein Hydrolysates | 1 YST | 2 YST, SOY | 2 YST, PRI | 3 YST, PRI, LAC | 5 YST, PRI, LAC, CAS, SOY | 3 YST, CAS, TPB |
| Other Additives | 0 | 1 Dextran | 0 | 0 | 4 Chitosan, Dextran, Glutathione, Glycerol | 0 |
| References | [20, 36, 37] | [40] | [32] | [8] | [41] | [42] |

*Acronyms/Abbreviations*: *CAS* casein hydrolysate, *LAC* lactalbumin hydrolysate, *PRI* primatone RL, *SOY* soy hydrolysate, *TPB* tryptose phosphate broth, *YST* yeast extract

[a]Original non-fortified version of SF1. The fortified version is supplemented with 10 % IP301 (derived from IPL41), additional amino acids (MET, CYS, ASN) and vitamins (Choline Chloride, Inosine)

[b]VPM3 formulation is modified from that reported [41], as a 6th hydrolysate (HyPep Dev 4602) is no longer included

[c]These nutrient groups in SF1 are assumed to be the same as ISFM, since the information is not clear in the reference [8]

PCDs (e.g., 8–11 × $10^6$ cells/mL) and to support better infections at high cell densities (Sf-9, Tn-5), by supplementing the medium with 10 % IP301 (a variant of IPL41), as well as methionine, cystine, asparagine, choline chloride, and inosine [8].

VPM3 (Virus Production Medium 3) was developed in our group in collaboration with Stefan Weiss [20, 43], specifically for HearNPV production in the HzAM1 cell line, for use as a bioinsecticide [41]. It contains five added amino acids (asparagine, cystine, glutamic acid, lysine, and methionine) and five hydrolysates (3 g/L yeast extract, 2.5 g/L Primatone RL, and 0.5 g/L each of soy hydrolysate, casein hydrolysate, and lactalbumin hydrolysate). The original VPM3 formulation [41] contained a 6th hydrolysate (HyPep Dev 4602) that is no longer included. VPM3 supported PCDs of up to 3–4 × $10^6$ cells/mL [34, 44], but since HzAM1 cells are more than double the volume of Sf-9 cells [45], the biomass supported by VPM3 is comparable to that of SF1 for Sf-9 cells. Subsequently, VPM3 was streamlined to two-hydrolysate variants using an extensive statistical optimization routine [34]. The most promising of these are YST-AMP (6.2 g/L yeast extract, 6.3 g/L meat peptone) and YST-POT (6.3 g/L yeast extract, 5.3 g/L potato peptone), which are just as efficacious as the original VPM3 and the commercial Sf-900 II SFM in supporting HearNPV production. This study demonstrated that two hydrolysates are probably the lower limit for low cost formulations with reduced added amino acids content, as one-hydrolysate versions of VPM3 are significantly worse in performance [34]. On the other hand, a

single hydrolysate is sufficient when a full complement of added amino acids is present, such as in ISFM [36, 37]. Protein hydrolysates derived from different animal/plant/microbial sources and different manufacturing processes are likely to exhibit divergent amino acid and oligopeptide profiles [46], hence it is not surprising that hydrolysate mixtures are better for formulations with reduced added amino acids.

Recently, UNL10 SFM was developed for AgMNPV production in the saUFL-AG-286 cell line, for use as a bioinsecticide [42]. It has 20 added amino acids (but most are at much reduced levels from that of classical basal media), three hydrolysates (3 g/L yeast extract, 3 g/L casein hydrolysate and 1 g/L tryptose phosphate broth), and a lipid emulsion containing soybean oil instead of cod liver oil for fatty acids supply. UNL10 can support PCDs of up to $3 \times 10^6$ cells/mL.

Among the LC-SFM discussed above, most have the advantage of being meat-free formulations, including UNL10, VPM3 YST-POT variant, ISYL, and YSD (a variant of YPR in which Primatone RL is replaced with soy hydrolysate [47]). However, none of these are Animal Component Free, (ACF), formulations, since UNL10 contains casein hydrolysate and the others use the conventional lipid emulsion containing cod liver oil. Furthermore, the added cholesterol may be of animal origin, e.g., if it is derived from sources such as sheep's wool. Completely ACF formulations are desirable to avoid regulatory concerns (e.g., for prions), which are feasible as plant-based hydrolysates and fatty acid sources are widely available. In addition, plant-based or synthetic (e.g., SyntheChol™, Sigma-Aldrich) sterols can also be specified.

The materials cost of the published LC-SFM can be estimated and compared on an equal footing, using the same cost analysis rationale described previously (calculations not shown). ISYL and YPR are the most expensive formulations, being in the range of $20/L. In contrast, SF1 (original and fortified), VPM3 (original and variants), and UFL10 are approximately half the cost of ISYL and YPR due to their reduced amino acids content. Of course, such prices are anticipated to be significantly lower if the media ingredients are purchased in bulk.

### 1.3 Chemically Defined Media

While SFM have many advantages over SCM for scale-up purposes, they share a common disadvantage, specifically, the lot-to-lot variability of undefined components. In the case of SFM, the two main undefined components are the protein hydrolysates and fatty acids source (e.g., cod liver oil). The protein feedstock and manufacturing process of each type of hydrolysate are subject to seasonal/temporal variability, which results in variability in the hydrolysate's chemical composition, which in turn affects the efficacy of the medium and the reproducibility of the cell culture process [29, 31, 48]. The problems associated with such variabilities are of course

enhanced in LC-SFM formulations, which rely on larger numbers and/or higher concentrations of hydrolysates. Ultrafiltration of hydrolysate solutions (10,000 MW cutoff) may help improve process reproducibility by removing endotoxins [31], but the underlying variability in chemical composition remains unresolved.

Another disadvantage of using hydrolysates is that nutrient delivery is untargeted, since both desirable and undesirable components have to be added together. For example, hydrolysates may contain a high level of inorganic salts [31], which contribute to high medium osmolality when elevated hydrolysate concentrations are required. Optimal growth of Lepidopteran insect cells is obtained only at a relatively narrow osmolality range of 350–385 mOsmol/kg [49]. In addition, certain components of hydrolysates may impart specific cell toxicity or growth inhibition properties [50]. For example, the toxic metabolite lactate can be present at significant levels (up to 7 % (w/w)) in yeast extract, possibly due to lactobacillus contamination since hydrolysate manufacturing processes are not aseptic [29, 31]. Lactate has been shown to be inhibitory for the growth of Bm5 (*Bombyx mori*) and Sf-9 cells [51, 52], and to reduce BV titers and heterologous protein production in rAcMNPV-infected Sf-9 cells [52]. The inefficiency in using hydrolysates is most apparent when intensifying the process from batch to fed-batch modes, since hydrolysate solubility, toxicity, and salt content will restrict the concentration factor of the nutrient feed.

Hence, a completely chemically defined medium (CDM), which is independent of hydrolysates, and is protein- and animal component-free, would be very beneficial for ICT, in terms of process reproducibility and scalability. Unfortunately, there are currently no commercially available CDM for insect cells. In contrast, commercial CDM are widely available for mammalian cells (e.g., CD OptiCHO™, Life Technologies; HyClone® CDM4CHO™, Thermo Scientific; PowerCHO™, Lonza; IS CHO-CD™, Irvine Scientific), which reflect the industrial importance of mammalian expression systems. Nevertheless, there are three fully disclosed insect cell CDM formulations in the literature (Table 1), but these are generally unoptimized, complex, and not well validated (being tested using poorly defined adherent cultures). Of these formulations, the older two (L21 and Wilkie's CDM) can support Lepidopteran cell growth [27, 53, 54], while the most recent (MTCM-1520 CDM) can only support Dipteran cell growth [55].

Based on the experience of mammalian cell cultures, it seems feasible that an optimized CDM can be developed that supports high insect cell densities and recombinant protein production, and be suitable for the scale-up of suspension culture-based ICT processes. In our laboratory, a semi-defined SFM is used routinely, consisting of IPL41 chemically defined basal medium, 1 % (v/v) chemically defined lipids, and only 1 undefined component being

4 g/L yeastolate ultrafiltrate (all components sourced from Life Technologies). This version of IPL41 SFM can support PCDs of up to $14 \times 10^6$ cells/mL (uninfected Sf-9 cells) and optimal recombinant ß-Gal yields of around 150,000 Units/$10^6$ cells (rAcMNPV-infected Sf-9 cells) (unpublished data), which are similar to the production capacities reported for Sf-900 II SFM [56, 57]. However, cell growth is nonexistent within three passages if the yeast extract is taken out (unpublished data), thereby illustrating the critical importance of this supplement in SFM. Hence, an insect CDM could be derived by replacing the growth proliferation factors of yeast extract with chemically defined substitutes, which may include efficient forms of chelated iron [58, 59], synthetic oligopeptides [60], trace elements such as sodium selenite [24, 61], antitoxic factors [24], and even commercial hydrolysate substitutes (e.g., EX-CELL™ CD Hydrolysate Fusion, Sigma-Aldrich; BD Recharge™, BD Biosciences). The identity of specific bioactive chemicals may be gleaned from reported mammalian cell CDM formulations such as CDSS [24], MET1.5 [59], and CD-CHO [58], as well as the insect cell CDM formulations discussed previously. The recent commercialization of ICT-expressed human and animal biologics [3] may spur the industrial development of Lepidopteran cell CDM in the near future.

### 1.4 Serum-Free Feeds

In general, media are designed to propagate cell cultures in batch-mode in which fixed volumes of medium and cell inoculum are added together to achieve the desired seeding cell density (SCD). Batch cultures are not supplied with additional nutrients, hence the culture volume is constant. Each type of medium supports a specific uninfected PCD, and a specific infected PCD range at which maximum volumetric yields (VY, Units/mL) are obtained (recombinant protein or virus product). In general, the optimum infected PCD range is in the vicinity of 50 % or less of the uninfected PCD (*see* Table 3), a phenomenon which is not yet fully explained, but which likely involves nutrient limitation. In fact, the VY of a baculovirus product is highly correlated to the PCD, which has been described as the "Cell Yield Concept" [56]. To illustrate this phenomenon, the VY initially increases linearly with increasing PCD, reaches a maximum at the optimum PCD, then declines rapidly at higher PCDs, as observed in previous investigations [42, 56, 57, 62, 63]. The specific yield (SY, Units/cell) may be relatively constant at lower cell densities, and only declines significantly at cell densities above the optimum PCD [56, 57, 63, 73], but a continuous decline in SY may also occur [42].

If higher VYs are desired, then the batch culture may be supplemented with additional nutrients in the fed-batch mode, which reverses the decline in productivity at higher PCDs, and hence extends the region in which a linear relationship exists between the VY and the infected PCD. In a typical fed-batch regime, a batch

culture is first setup and grown to the desired feeding cell density (FCD), at which feeding is initiated. The feed addition may be a single or multiple event, pulsed or continuous in mode, resulting in an increase in the culture volume [64–66]. Serum-free feeds (SFF) are generally more concentrated versions of SFM, and may be streamlined to include only the most critical ingredients such as hydrolysates, added amino acids, sugars, and lipids. Feeds are designed as concentrates so that dilution of the cell culture is minimized, which is especially important if basal components such as inorganic salts and vitamins are excluded from the feed.

Conventional SFF are in general not low-cost formulations (due to high numbers or high concentrations of added amino acids), and are multicomponent in structure (i.e., multiple feeds representing different nutrient groups). Over the past 20 years, significant progress has been made in designing efficacious fed-batch systems for ICT, mostly using conventional SFF and involving the Sf-9/rAcMNPV expression system (Table 5). One of the earliest and simplest SFF designs is by Nguyen et al. [67], which only contains yeast extract, lipid emulsion, glutamine and glucose, and which supports a reasonable uninfected PCD of $12 \times 10^6$ cells/mL (Sf-9 cells). Another early SFF is NCC1 by Bedard et al. [64], which contains yeast extract, 18 added amino acids, lipid emulsion, vitamins, trace elements, and glucose. The NCC1 feed is optimized using factorial experiments, and is able to support uninfected PCDs of up to $23 \times 10^6$ cells/mL (Sf-9 cells), and maximum recombinant ß-Galactosidase (ß-Gal) VYs up to an infected PCD of $7 \times 10^6$ cells/mL (rAcMNPV/Sf-9 system), by using a single pulsed feeding regime [64]. Bedard et al. [68] further derived an improved feed (NCC4) with fourfold more yeast extract and 19 added amino acids, which could support uninfected PCDs of $28 \times 10^6$ cells/mL and peak ß-Gal VYs at $11.5 \times 10^6$ cells/mL. The NCC4 feed is capable of even higher performance when a semi-continuous feeding regime is implemented, thereby resulting in uninfected PCDs of $52 \times 10^6$ cells/mL and peak ß-Gal VYs at $14 \times 10^6$ cells/mL [66]. The NCC4 fed-batch regime has been used to produce both secreted and non-secreted products, including SEAP, gene therapy vectors (rAAV), and influenza vaccines (rHA) (Table 5).

Another conventional SFF is the one developed by our group (Chan et al. [65]), which is optimized using factorial and response surface experiments, that contains yeast extract, 20 added amino acids, lipid emulsion, and glucose. Like NCC1, this feed is added as a single pulsed feed, and could support uninfected PCDs of up to $20 \times 10^6$ cells/mL (Sf-9 cells), and peak recombinant protein VYs (secreted and non-secreted) at an optimum infected PCD range of $7–14 \times 10^6$ cells/mL (Sf-9/rAcMNPV system) [62, 65].

Two other SFF systems have been developed, i.e., Chiou's [69] and Marteijn's [45] Feeds, but these are unusual in that the batch cultures are propagated in serum-containing medium, unlike

**Table 5**
**Performance of a selection of serum-free feeds (SFF), when used to support Lepidopteran insect cell growth and baculovirus infection in fed-batch suspension cultures**

| | Feed | Medium | Cell line | Baculovirus | FCD ($10^6$ cells/mL) | Uninfected PCD ($10^6$ cells/mL) | Maximum protein yield (PCD) (various units) ($10^6$ cells/mL) | References |
|---|---|---|---|---|---|---|---|---|
| 1 | Nguyen's Feed | EX-CELL™ 400 | Sf9 | rAcMNPV | 3 (5 feeds) | 12 | 20 mg/L rhNGF (7.5) | [67] |
| 2 | NCC1 | Sf-900™ II | Sf9 | rAcMNPV | 7 (1 feed) | 16–23 | 241 U/mL β-Gal (7) | [64] |
| 3 | NCC4 | Sf-900™ II | Sf9 | rAcMNPV | 5 (1 feed) | 28 | 1100 U/mL β-Gal (11.5) | [68] |
| | | Sf-900™ II | Sf9 | rAcMNPV | 4.5 (≥3 feeds) | 52 | 372 U/mL β-Gal (14) | [66] |
| | | Sf-900™ II | Sf9-SEAP | Not infected | 8 (7 feeds) | 28 | 11.6 IU/mL SEAP (28) | [70] |
| | | Sf-900™ II | Sf9 | rAcMNPV | 3 (3 feeds) | – | $2.4 \times 10^9$ ETU/mL rAAV (9.4) | [72] |
| | | PSFM | expreSF® + | rAcMNPV | 4 (2 feeds) | - | 61.6 mg/L rHA (8.6) | [71] |
| 4 | Chan's Feed | Sf-900™ II | Sf9 | rAcMNPV | 3 (1 feed) | 20 | $12–16 \times 10^5$ U/mL β-Gal (7–11) | [65] |
| | | Sf-900™ II | Sf9 | rAcMNPV | 3.5 (1 feed) | 20 | 10–12 mg/L NS1 (10–14) | [62] |
| 5 | Chiou's Feed | TNMFH +10 % FBS | Sf21 | rAcMNPV | 0.5 (7 feeds) | >19 | $3.5 \times 10^6$ U/mL hIL-5 (17) | [69] |
| 6 | Marteijn's Feed | HyQ-CCM3 +2 % FBS | HzAM1 | Not infected | ~0.5 (7 feeds) | 19.5 | Not infected | [45] |

*Acronyms/Abbreviations*: *FBS* fetal bovine serum, *FCD* feeding cell density, *PCD* peak cell density
*Insect cell lines*: expreSF® + (derived from Sf9), HzAM1 (*Helicoverpa zea*), Sf9 (*Spodoptera frugiperda*), Sf21 (*S. frugiperda*)

the other examples mentioned above (Table 5). Chiou et al. [69] optimized a feed based on the stoichiometric coefficients of key nutrients. This feed consists of yeast extract, 19 added amino acids, and glucose, but lipid emulsion is excluded, presumably because the serum-derived lipids are deemed to be sufficient. Nevertheless, Chiou's Feed could support uninfected PCDs of $>19 \times 10^6$ cells/mL (Sf-21 cells), and peak recombinant protein VYs at $17 \times 10^6$ cells/mL (Sf-21/rAcMNPV system), which are among the highest reported. Marteijn et al. [45] employed a novel method (genetic algorithms) to optimize a feed containing three hydrolysates (yeast extract, wheat-gluten hydrolysate, and lactalbumin hydrolysate), eight added amino acids, lipid emulsion, and glucose. Marteijn's Feed is able to support high uninfected PCDs of HzAM1 cells ($19.5 \times 10^6$ cells/mL), but this requires the addition of aseptically treated powders of hydrolysates, amino acids, and glucose (to avoid solubility constraints), which appear to be impractical for large-scale processes.

In summarizing this overview of conventional SFF, it is clear that fed-batch regimes can provide significant improvements in productivity when compared to batch regimes. For example, Sf-9 batch cultures grown in Sf-900 II™ SFM can only support uninfected PCDs of approximately $10 \times 10^6$ cells/mL and optimum infected PCDs of approximately $3–4 \times 10^6$ cells/mL (Table 3). However, fed-batch cultures are still subject to the cell density effect, as the region in which the VY increases linearly with respect to the PCD cannot be extended indefinitely, hence dramatic declines in VY are observed above a specific optimum PCD [62, 65, 68]. Many of these fed-batch regimes have also been tested in bench-scale stirred-tank bioreactors [45, 66, 67, 70–72], demonstrating the scalability of such processes.

While a significant number of LC-SFM have been described in the literature, there are as yet no reports on low-cost serum-free feeds (LC-SFF) to the best of our knowledge. Marteijn's Feed [45] approaches that of a low cost formulation, due to its reduced number of added amino acids, and the use of three different protein hydrolysates at high concentrations. However, this feed still contains a relatively high concentration of added amino acids, requires a powdered formulation that is difficult to scale, and has not been tested for baculovirus-infections.

### 1.5 Feed Development Challenges

Fed-batch systems for ICT have an inherent inefficiency since the feed has to support at least double the cell yield required in the (infected) production process, which is not the case for mammalian cell manufacturing systems [74]. This inefficiency magnifies the challenges of feed design, which are subject to the solubility, osmolality, and toxicity constraints imposed by each feed component. Such challenges would be even greater in low-cost systems, which have a higher reliance on undefined and variable protein hydrolysates.

It is not clear why Lepidopteran insect cells cannot be infected successfully at the highest cell yields supported by a particular medium or feed, but infection-specific nutrient limitation may be involved. Extracellular free amino acid analysis of optimally infected batch Sf-9 cultures (delivering peak VYs) showed that Cystine is depleted [57]. However, another study showed that free amino acids are not depleted in both optimally infected batch and fed-batch Sf-9 cultures, although Cystine is significantly consumed in the fed-batch case [75]. Furthermore, energy substrates (e.g., glucose) are not depleted in such studies. However, the limiting substrate may not be a macronutrient, and therefore may be difficult to define.

As the cell density increases, toxic metabolites may also accumulate to levels that result in suboptimal infections. This could partly explain the improvement in VY when spent medium is exchanged with fresh medium prior to infection [57]. Sf-9 cells generally do not accumulate high levels of lactate and are not as sensitive to ammonia as mammalian cells [76–78]. For example, lactate levels in high density fed-batch infections ($14–17 \times 10^6$ cells/mL, Sf9/rAcMNPV) can be as low as 3–5 mM [66]. However, other insect cell lines, e.g., Tn-5, may exhibit higher lactate production [79].

In reality, an ICT production process involves complex interactions between the cell line, virus and medium. Hence, identifying the basis of the cell density effect in ICT may be very difficult without a fundamental approach such as systems biology. Our group has embarked on metabolomics and transcriptomics studies [80, 81] with this approach in mind. It is anticipated that such studies will assist in the design of more efficient and targeted feeding regimes (chemically defined or hydrolysates based) that enable insect cells to be infected successfully at higher densities, and hence to deliver further improvements in recombinant protein and wild-type baculovirus yields. Recent metabolic flux studies of uninfected Sf-9 cells show that the central carbon metabolism is inhibited at increased cell densities, thereby demonstrating the utility of this approach [82].

## 2 Materials

### *2.1 Cell Culture Equipment*

#### *2.1.1 Cell Culture (Suspension Cell Culture)*

1. Biological Safety Cabinet (BSC), (Class II).
2. Refrigerated incubator shaker.
3. Orbital shaker platform.
4. Erlenmeyer flask with screw cap (autoclavable glass and/or disposable (e.g., polycarbonate)), 125 and 250 mL.
5. Fernbach flask with screw cap (autoclavable glass and/or disposable (e.g., polycarbonate)), ~3 L.

6. 50 mL tube bioreactor (e.g., TubeSpin® Bioreactor 50 with filter screw cap, Techno Plastic Products).
7. Serological pipets: 1, 2, 5, 10, 25, 50 mL single-use presterilized polystyrene.
8. Pipet gun (e.g., Pipet-Aid®, Drummond Scientific).

*2.1.2 Cell Counts/ Sampling*

1. Manual cell counts: phase-contrast microscope (e.g., BX43 upright microscope, Olympus), improved-Neubauer hemocytometer, and 2-key manual cell counter (i.e., click counter).
2. Automated cell counts (e.g., Multisizer™ 4 Coulter Counter or ViCell® Cell Viability Analyzer, Beckman Coulter).
3. Refrigerated centrifuge.
4. Vortex mixer.
5. Single-channel adjustable pipettes: 10, 20, 100, 200, and 1000 μL (e.g., PIPETMAN Classic™, Gilson, Inc.)
6. Pipette tips.
7. Microcentrifuge tubes.
8. Centrifuge tubes, 15 and 50 mL (e.g., single-use presterilized polypropylene tubes with screw cap, Corning).
9. Diluent for cell counts (same medium as that used to propagate cells).
10. Trypan Blue (0.4 % (w/v) solution).

***2.2 Cell Cryopreservation Equipment***

1. Cryogenic dewar (e.g., LD50, 50 L capacity, liquid nitrogen storage, Taylor-Wharton).
2. Ultralow temperature freezer (e.g., VIP™ MDF-U55V, −86 °C, Panasonic).
3. Freezing container (e.g., Mr Frosty™ Freezing Container, Thermo Fisher Scientific).
4. Cryogenic vials (e.g., 1 mL Nunc Cryotubes™, Thermo Fisher Scientific).
5. Cryogenic vial protection (e.g., Nunc Cryoflex™ Tube Wrap, Thermo Fisher Scientific).
6. Spirit lamp and scissor (hemostatic) clamps.
7. Isopropyl alcohol (≥99.7 %).
8. Dimethyl sulfoxide (DMSO) (sterile-filtered, ≥99.7 %, e.g., Hybri-Max™, Sigma-Aldrich).

***2.3 Serum-Free Medium/Feed Preparation Equipment***

1. Biological Safety Cabinet (Class II).
2. Purified water system (Type 1 cell-culture-grade delivering 18.2 MΩ-cm resistivity, e.g. Milli-Q® Integral, Merck-Millipore).
3. pH meter.

4. Osmometer (e.g., Vapro® 5600 Vapor Pressure Osmometer, Wescor).
5. Weighing balances (e.g., XS105 Dual Range Analytical Balance, 0.00001 g and MS1003S Precision Balance, 0.001 g, Mettler Toledo).
6. Low flowrate peristaltic pump (e.g., Masterflex®, 0.8–3 mm ID silicone tubing, 140–4000 μL/min, Cole-Palmer).
7. High flowrate peristaltic pump (e.g., Masterflex® I/P® digital modular drive/Easy-Load® pump head, Pharmed® BPT 0.25″ID tubing, 0.12–17 L/min, Cole-Palmer).
8. Small-scale mixing (1–10 L) (magnetic stirring platform and stir bars).
9. Large-scale mixing (10–40 L) (e.g., 50–2000 rpm, with stainless steel shaft and 6-blade Rushton impeller).
10. Large water bath, with ~80 L total volume.
11. Heating immersion circulator with ~15 L/min pump rate.
12. High pressure homogenizer for lipids emulsification (optional).
13. Measuring cylinders (graduated), 0.05, 0.1, 0.25, 0.5, 1 and 2 L.
14. Small-scale media preparation vessels: 0.1–10 L Duran® glass bottles with screw cap (Schott, Mainz, Germany). Use wide-mouth (80 mm) bottles for the larger volumes.
15. Large-scale medium/feed preparation vessels, 20–50 L.
16. Storage bottles with screw cap, 1 L.
17. Sample containers (e.g., 70 mL single-use presterilized polycarbonate with screw cap).
18. Small-scale liquid sterilization (e.g., Minisart® NML single-use syringe filter, 0.2 μm, Sartorius, with 50 mL needle-free syringe).
19. Large-scale liquid sterilization (e.g., Sartobran® P 300 single-use membrane filter capsule, 0.45 μm prefilter, 0.2 μm endfilter, 0.1–50 L of filtrate, Sartorius).

### 2.4 Serum-Free Medium/Feed Preparation Requirements

1. All water used in medium or feed preparation must be Type 1 purified water of 18.2 MΩ-cm resistivity, at room temperature. This includes any water that ends up in the reconstituted medium or feed, e.g., stock solutions, buffers, rinse solutions, and water used to fill wash bottles and rinse medium/feed preparation vessels and accessories.
2. All medium/feed preparation vessels and accessories (bottles, carboys, measuring cylinders, spatulas, stir bars, wash bottles, pump tubing, mixer shaft/impeller, etc.) must be depyrogenated and rinsed with purified water prior to use (*see* Subheading 3.3.1), unless they are presterilized single-use items.

3. Weigh out medium/feed chemicals and ingredients in individual 70 mL single-use screw-capped sample containers, using a balance with either 0.001 g or 0.00001 g readability (depending on the amount required), and stainless steel spatulas. Each container is then labelled with the name and weight of the ingredient (*see* **Note 1**).
4. For the preparation of all stock solutions, media and feeds, the ingredients should be dissolved in the order indicated, using purified water or analytical-grade ethanol. Each ingredient is allowed to dissolve for 3–5 min prior to adding the next ingredient, in the magnetically stirred preparation vessel. As a rule, the next ingredient is only added when the previous ingredient is fully dissolved by eye, hence the mixing times have to be adjusted accordingly (*see* **Note 2**).
5. Stock solutions, media, and feeds are made up to the final volume (with purified water or analytical-grade ethanol), using the appropriate-sized measuring cylinder. For the preparation of larger volumes of media and feeds (e.g., >2 L), the preparation vessel is manually calibrated with 0.5 L or 1 L graduations using a measuring cylinder, as the factory-engraved graduations may not be accurate. Alternatively, final volumes may be made up by weight.
6. Chemicals must be at least analytical grade, and are preferably cell-culture grade. An example of a chemical supplier is Sigma-Aldrich.
7. Protein hydrolysates: yeast extract (e.g., Hy-Yest™ 444, Kerry Bio-Science), meat extract (e.g., Primatone™ RL, Kerry Bio-Science), Lactalbumin Hydrolysate (e.g., Sigma-Aldrich), Casein Hydrolysate (e.g., N-Z-Amine™ A, Kerry Bio-Science), Soy Hydrolysate (e.g., Hy-Soy™, Kerry Bio-Science).

### 2.5 Stock Solutions

1. 4 M HCl: 33.3 mL 12 M HCl, 66.7 mL water.
2. 4 M NaOH: Dissolve 16 g NaOH in 100 mL water.
3. 0.1 % (v/v) Trypan Blue: 2.5 mL 0.4 % (w/v) Trypan Blue stock solution, 7.5 mL of the medium in use.
4. 15 % (v/v) DMSO: 1.5 mL DMSO, 8.5 mL of the medium in use.
5. 70 % (v/v) Ethanol (for sanitization): 700 mL Absolute Ethanol, 300 mL water.
6. 1 % (w/v) Terg-A-Zyme® (for cleaning): 100 g Terg-A-Zyme® detergent (Alconox), 10 L hot tap water (55 °C) in a plastic tub.
7. 0.1 M NaOH (for depyrogenation): Dissolve 40 g NaOH in 10 L tap water in a plastic tub.

### 2.6 Commercial Serum-Free Media (Selection Only)

Basal media require supplementation with lipid emulsion and yeast extract/hydrolysates for the support of insect cell growth and baculovirus production:

#### 2.6.1 Basal Media

1. Grace's: liquid or powder (e.g., Life Technologies).
2. IPL-41: liquid (e.g., Life Technologies).

#### 2.6.2 Supplements

1. Chemically Defined Lipid Concentrate: liquid (e.g., Life Technologies).
2. Chemically Defined Lipid Mixture 1: liquid (e.g., Sigma-Aldrich).
3. Lipid Medium Supplement: liquid (e.g., Sigma-Aldrich).
4. Yeastolate Ultrafiltrate 50×: liquid (e.g., Life Technologies).
5. 5 M NaCl: Dissolve 29.22 g NaCl in water and make up to 100 mL. Filter-sterilize and store at 4 °C for up to 1 year.

#### 2.6.3 Complete Media

1. EX-CELL™ 405: liquid or powder (Sigma-Aldrich).
2. EX-CELL™ 420: liquid or powder (Sigma-Aldrich).
3. Express Five®: liquid (Life Technologies), requires Glutamine supplementation.
4. Sf-900™ II: liquid (Life Technologies).
5. Sf-900™ III: liquid (Life Technologies).
6. SFM4-Insect™ HyClone® (Thermo Scientific).
7. SFX-Insect™ HyClone® (Thermo Scientific).

### 2.7 Stock Solutions for a Conventional Serum-Free Feed (Chan's Feed)

1. Amino acid 1 concentrate (soluble): dissolve 9.929 g L-Aspartic Acid · K, 11.787 g L-Glutamic Acid · K · $H_2O$, 3.265 g trans-4-Hydroxy-L-Proline, 9.574 g L-Serine, 6.566 g L-Asparagine, 3.108 g Glycine, 22.769 g L-Glutamine, 3.123 g L-Histidine · HCl · $H_2O$, 1.977 g L-Threonine, 7.689 g L-Arginine · HCl, 4.202 g L-Proline, 1.160 g L-Valine, 0.985 g L-Methionine, 4.342 g L-Isoleucine, 2.177 g L-Leucine, 3.287 g L-Phenylalanine and 6.959 g L-Lysine · HCl in water and make up to 1 L. Filter-sterilize and store in convenient aliquots at −20 °C, indefinitely.
2. Amino acid 2 concentrate (insoluble): dissolve 12.624 g L-Tyrosine · HCl, 12.713 g L-Cystine · 2HCl, and 5.433 g L-Tryptophan in 4 M HCl, then make up to 1 L with water (pH of <1). Filter-sterilize and store in convenient aliquots at −20 °C, indefinitely.
3. Glucose concentrate: dissolve 504.45 g D-Glucose in water and make up to 1 L (2.8 M). Filter-sterilize and store at 4 °C, indefinitely.
4. Vitamin concentrate: dissolve 47 mg Thiamine · HCl, 45 mg Riboflavin, 2 mg D-Pantothenic Acid Hemicalcium Salt, 232 mg

Pyridoxine·HCl, 185 mg 4-Aminobenzoic Acid, 92 mg Nicotinic Acid, 232 mg myo-inositol, 93 mg Biotin, 11,569 mg Choline Chloride, 136 mg Vitamin $B_{12}$, and 49 mg Folic Acid in water and make up to 1 L. Filter-sterilize and store at 4 °C, indefinitely.

5. Trace element concentrate: dissolve 25 mg $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 29 mg $CoCl_2\cdot 6H_2O$, 116 mg $CuCl_2\cdot 2H_2O$, 12 mg $MnCl_2\cdot 4H_2O$, 23 mg $ZnCl_2$, 320 mg $FeSO_4\cdot 7H_2O$ in water and make up to 1 L. Filter-sterilize the Trace Element Solution and store at 4 °C, indefinitely.

## 3 Methods

### 3.1 Serum-Free Media Preparation

#### 3.1.1 Preparing 10 L Commercial Serum-Free Medium from a Powder Formulation

This procedure is suitable for reconstituting powdered medium formulations, including those for basal (e.g., Grace's, IPL-41) and complete (e.g., EX-CELL 420) SFM designed for Lepidopteran insect cell growth.

1. Place the medium preparation vessel (10 L widemouth glass bottle) on a magnetic stirrer, add a stir bar.
2. Fill the vessel with 9 L water, and start mixing the liquid (increase the stir speed until a slight vortex is formed).
3. Carefully introduce the medium powder (e.g., 10 × 1 L lots) into the vessel, with minimal airborne escape of powder.
4. Rinse each medium container (packaging) with some water from a wash-bottle to dissolve any leftover powder. Add the washing liquid to the preparation vessel.
5. Stir the reconstituted medium until it is completely dissolved, e.g., 2 h.
6. Add any required supplements according to the manufacturer's instructions (e.g., $NaHCO_3$), and stir for another 0.5 h.
7. Adjust the medium's pH to 6–6.3 with 4 M HCl or 4 M NaOH.
8. Adjust the medium to a final volume of 10 L with water.
9. Adjust the medium's osmolality to 310–340 mOsmol/kg for basal medium, and 355–375 mOsmol/kg for complete medium, using NaCl.
10. Stir the reconstituted medium for a total mixing time of 6–7 h before sterile-filtration.
11. In a BSC, filter-sterilize the medium using a 0.2 μm Sartobran® P 300 filter capsule into sterile 1 L glass or single-use polycarbonate bottles (*see* Subheading 3.3.4), and store at 4 °C for 2–3 months.
12. Basal medium requires supplementation with additional nutrients prior to use, e.g., for IPL-41 basal medium, add 20 mL/L

50× yeastolate ultrafiltrate (4 g/L) and 10 mL/L chemically defined lipid concentrate (0.0045 g/L cholesterol). Adjust the osmolality to 355–375 mOsmol/kg with sterile 5 M NaCl solution.

*3.1.2 Preparing a Lipid Emulsion (80×)*

This method of preparing a lipid emulsion is a scaled-up version of Inlow's method [36]. The lipid emulsion is suitable for addition to LC-SFM formulations such as VPM3 prior to filter sterilization. Alternatively, the lipid emulsion can be filter-sterilized separately and used as a supplement for basal media (store at 4 °C and use within 1–2 months).

1. Set up the large water bath: place an 80 L tank on a trolley beside the sink, fill with deionized (e.g., reverse osmosis) water to about 80 % volume, and install the heating immersion circulator at a setpoint of 37 °C (*see* **Note 3**).
2. Park the trolley by a workbench.
3. Carefully place an empty 2 L measuring cylinder into the water bath, and immobilize it at the bottom with a heavy object. The top of the cylinder should be well above the water line (*see* **Note 4**).
4. Install the overhead stirrer on a stand, position the drive shaft at the central axis of the measuring cylinder, and adjust the height of the Rushton impeller so that it is approximately 1 cm above the base of the cylinder.
5. Pour the Pluronic solution (562.5 mL) into the measuring cylinder, being careful to minimize foam formation.
6. Hang the bottle of Lipids-Ethanol solution (62.5 mL) in the water bath using a rack.
7. Allow the Lipids-Ethanol and Pluronic solutions to warm up to 37 °C (check with a thermometer).
8. Turn on the overhead stirrer at 550 rpm, so that a strong, uniform, and stable vortex is formed in the Pluronic solution.
9. Place a length of silicone tubing into the lipids-ethanol solution at one end, and above the Pluronic vortex at the other end, and install the tubing into a low flow rate peristaltic pump.
10. Start the pump, and slowly drip the lipids-ethanol solution onto the angled side of the Pluronic vortex, at a flowrate of 0.5 mL/min.
11. The lipids emulsification procedure takes approximately 2 h to complete, which may be followed by a micro-emulsification step using a high pressure homogenizer (optional).
12. Measure the final volume (FV) of the lipid emulsion using a 1 L measuring cylinder, as the final volume may be less than 625 mL (V) due to evaporation.

13. Transfer the lipid emulsion into a 1 L glass bottle, overlay with $N_2$, and store at room temperature and use within 1–2 days (added to VPM3 medium prior to filter sterilization).

### 3.2 Serum-Free Feed Preparation

#### 3.2.1 Preparing a Conventional Serum-Free Feed (Chan's Feed)

Conventional SFF for insect cell fed-batch cultures are usually multicomponent, each representing a different nutrient group (amino acids, carbohydrates, lipids, trace elements, vitamins, and most importantly protein hydrolysates), which mirror the composition of the medium, but are formulated at much higher concentrations. The following SFF concentrates have worked well in our group for the Sf-9/rAcMNPV system, designated as Chan's Feed [65], including the Essential Feeds (50× Yeastolate Ultrafiltrate, Chemically Defined Lipid Concentrate, Amino Acid 1 Concentrate, Amino Acid 2 Concentrate, and Glucose Concentrate) and the Non-essential Feeds (Vitamin Concentrate and Trace Element Concentrate), as described in Subheadings 2.6.2 and 2.7. These Feeds are similar to other conventional SSF reported in the literature (Table 5).

### 3.3 General Cell Culture Techniques

#### 3.3.1 Depyrogenation of Labware

Pyrogens in the context of cell culture refer to lipopolysaccharides from bacterial cell walls, which exhibit cellular toxicity (endotoxin). Pyrogens may be present on surfaces even from low-level bacterial contamination, hence media preparation vessels and accessories should be depyrogenated prior to use, using the following procedure:

1. Soak the items in 1 % (w/v) Tergazyme® solution at 55 °C for 24 h, then rinse thoroughly with hot tap water.
2. Depyrogenate the items by soaking them in 0.1 M NaOH solution for 24 h. Then rinse thoroughly with hot tap water, followed by deionized (e.g., reverse osmosis) water, and finally purified water.
3. Dry the items on a rack prior to usage or short-term storage.
4. Autoclavable glass and plastic items may be sterilized in the autoclave at 121 °C (45–60 min) for long-term storage if they are suitably covered (e.g., loosened screw cap for flasks/bottles, sealed paper bag for other items).

#### 3.3.2 Aseptic Technique in the Biological Safety Cabinet (BSC)

1. Aseptic technique is critical for insect cell cultures as the risk of microbial contamination is high due to the richness of the growth media and the slow growth rate of insect cells in comparison to that of most microbes (24 h vs. ≤1 h doubling times). Insect cell culture experiments, uninfected or baculovirus-infected, can take up to 2–3 weeks to complete depending on scale and mode (batch or fed-batch).
2. The sterility of insect cell cultures is best managed inside a Class II BSC and by using pre-sterilized single-use plastic

accessories (e.g., culture flasks, serological pipettes, filters, bottles and centrifuge tubes) as much as possible. If reusable items are employed, then these should be well-cleaned, depyrogenated, and autoclave-sterilized.

3. Before working in a BSC, the cabinet bench and any items placed on it should be surface-sanitized to reduce the microbial load (e.g., by wiping with paper towels soaked in 70 % (v/v) ethanol).
4. When performing liquid handling procedures in a BSC, a high degree of attention to detail is required to maintain asepsis: (a) HEPA-filtered sterile air flows from the top to the bottom of the cabinet; thus, a sterile item (liquid or solid) remains sterile if care is taken not to pass a non-sterile item above it and physical contact between the sterile item and any non-sterile entity is avoided (solids, liquids, gases, or aerosols). (b) Good aseptic technique includes having a non-cluttered work area, leaving sufficient empty space between sterile objects, and a spatial memory of where the sterile and non-sterile items are situated.
5. The BSC should be decontaminated and NATA-tested annually to maintain optimal performance.

*3.3.3 Filter-Sterilize Solutions (Small Scale, Up to 50 mL)*

1. In a BSC, draw the desired volume of non-sterile solution into a 50 mL needle-free syringe. Aseptically attach a 0.2 μm Minisart® syringe filter onto the tip of the syringe.
2. Aseptically uncap a 50 mL centrifuge tube and place the sterile end of the filter directly on the mouth of the tube (angled position).
3. Carefully depress the syringe plunger to push the liquid through the filter (*see* **Note 5**).
4. When all the sterile filtrate has been collected, aseptically recap the tube.

*3.3.4 Filter-Sterilize Solutions (Large Scale, Up to 50 L)*

1. Place 1 L sterile storage bottles in the BSC, with the screw caps loosened.
2. Install a 2 m length of Pharmed® BPT tubing onto an Easy-Load® pump head connected to a Masterflex® I/P® pump drive.
3. Immerse the upstream end of the tube in the non-sterile solution and connect the other end to the hose barb of a Sartobran® P 300 filter capsule in the BSC secured using cable ties (keep most of the filter inside its packaging during this procedure).
4. Hold the filter capsule in one hand with the filter bell facing downwards in an angled position and then remove the packaging completely.

5. With the other hand carefully remove the filter bell cover, aseptically remove the cap of a bottle, and position the mouth of the bottle below the filter bell (angled, followed by upright position, for both filter and bottle).
6. Loosen the upstream filter air vent, start the pump at 0.15 L/min to fill up the filter capsule, and then tighten the air vent.
7. Once the filtrate starts flowing out into the bottle the pump speed can be increased to 0.3–0.5 L/min.
8. When 1 L of filtrate has been collected in the bottle, stop the pump, move the filled bottle to one side, and position another opened bottle below the filter. Then aseptically recap the filled bottle (*see* **Note 6**).
9. Repeat this procedure until all the solution has been filtered.

### 3.3.5 Batch Suspension Cell Culture

The following insect cell culture procedures (both batch and fed-batch modes) are focused on shaker flask suspension cultures. Techniques for adherent cultures, adapting adherent cultures to suspension cultures, and bioreactor-based suspension cultures, are provided in other chapters of this publication. Furthermore, baculovirus infection procedures are only briefly mentioned since they are covered in more detail in other chapters.

The stock cells are assumed to be fully adapted to serum-free suspension cultures. In general, SCM-based suspension cultures are readily adapted to SFM with minimum weaning, provided that the latter can support high cell densities, 24 h cell doubling times, and optimal wild-type baculovirus or baculovirus-expressed recombinant protein production.

Insect cell suspension cultures are initiated from cryopreserved stock cells (Subheadings 3.3.8 and 3.3.9). Once thawed, the cells are serially passaged regularly (e.g., twice weekly) in fresh SFM as Erlenmeyer shaker flask batch cultures. The following cell passaging procedure is suitable for Sf-9 cells grown in an optimized SFM such as Sf-900 II. Small-scale 125 mL shaker flasks (25–50 mL working volume) are used to save on medium costs. If larger volumes are required, then 250 mL shaker flasks (50–100 mL working volume) or 3 L Fernbach flasks (500–1000 mL working volume) can be used.

1. Sf-9 stock cells are ready for passaging when they have reached the mid-exponential growth phase (medium-dependent). Sf-9 cultures grown in Sf-900 II SFM reach a PCD of $>10^7$ cells/mL in our hands with a mid-exponential density of around $4–6 \times 10^6$ cells/mL with a cell viability of >95 % (*see* **Note 7**).
2. On the passaging day set up a new 25 mL culture (in a 125 mL flask) at a seeding density of $4–5 \times 10^5$ cells/mL, which represents an approximate tenfold dilution of the stock cells with fresh SFM (e.g., 2.5 mL cells + 22.5 mL SFM).

3. Install the flask on an orbital shaker platform (120 rpm) in a refrigerated incubator (27–28 °C). Ensure that the screw cap is loosened (e.g., quarter turn anticlockwise) to allow for gas exchange.
4. Allow the new stock cells to reach mid-exponential growth phase and then repeat the cell passaging procedure.
5. For a twice-weekly passaging routine, the following schedule works well for us (assuming a 24 h cell doubling time): *Monday*: Set up stock cells at $4 \times 10^5$ cells/mL (Passage N), *Friday*: Stock cells grown to $6 \times 10^6$ cells/mL (Passage N), set up new stock cells at $5 \times 10^5$ cells/mL (Passage N + 1), *Monday*: Stock cells grown to $4 \times 10^6$ cells/mL (Passage N + 1), set up new stock cells at $4 \times 10^5$ cells/mL (Passage N + 2), and so forth.
6. For cell lines that do not grow to as high a PCD as Sf-9 cells passaging is performed at lower cell densities. For example, HzAM1 cells are about double the size of Sf-9 cells and only grow to a PCD of $3–4 \times 10^6$ cells/mL in VPM3 LC-SFM [34]. In this case, seeding densities of $3 \times 10^5$ and $4 \times 10^5$ cells/mL are applied, on Monday and Friday, respectively. These relatively low seeding densities did not result in a significant lag phase in our laboratory.
7. The procedure used to set up experimental batch cultures is the same as that used for serial passaging of stock cells. The same agitation speed can be applied for both 125 mL and 250 mL shaker flasks (120 rpm). Lower agitation speeds (100–120 rpm) may be required for 3 L Fernbach flasks.
8. If the batch cultures are to be infected with baculovirus, then the settings of certain key infection parameters have to be optimized, including the infection cell density (ICD), multiplicity of infection (MOI), and PCD.
9. For the production of recombinant proteins using the Sf-9/rAcMNPV/Sf-900 II system, typical settings for ICD and MOI are $2–4 \times 10^6$ cells/mL and 5–10 PFU/cell, respectively. Maximum volumetric yields are obtained at PCDs of $3–5 \times 10^6$ cells/mL.
10. For the production of occlusion bodies (OBs) using the HzAM1/HearNPV/VPM3 system, typical settings for ICD and MOI are $0.5–1 \times 10^6$ cells/mL and 5–10 PFU/cell, respectively. Maximum volumetric yields are obtained at PCDs of $1–1.5 \times 10^6$ cells/mL.
11. For high-throughput experimental designs (e.g., statistics-based factorial designs), tube cultures are viable alternatives to shaker flask cultures, as demonstrated for Sf-9 [83] and HzAM1 [34] cells. In our laboratory, 50 mL TubeSpin® bioreactors with filter screw caps are employed at a working volume

of 10 mL and an agitation speed of 240 rpm. The tubes are installed on a plastic rack with a capacity for 20 × 50 mL tubes, which is secured tightly on an orbital shaker platform.

### 3.3.6 Fed-Batch Suspension Cell Culture: Conventional Feed

Fed-batch cultures are set up initially as batch cultures. When the cell density has reached the desired feeding cell density (FCD), the SFF is added as pulsed additions, either once or multiple times over several days. The procedure below describes a fed-batch production process for recombinant proteins with rAcMNPV-infected Sf-9 cells through the use of a conventional multicomponent SFF designed by our group (Chan's Feed [65], Table 5).

1. Set up a 100 mL Sf-9 culture (in a 250 mL flask) at 4–5 × $10^5$ cells/mL in Sf-900 II SFM.
2. Incubate the batch culture for 3 days to reach a FCD of 3–4 × $10^6$ cells/mL, then add the SFF, e.g., 4.6 mL 50× Yeastolate Ultrafiltrate, 1.1 mL Chemically Defined Lipid Concentrate, 7.5 mL Amino Acid 1, 2.2 mL Amino Acid 2, and 0.7 mL Glucose Concentrate.
3. Split the fed-batch culture equally into two shaker flasks and incubate for at least 1 day post feeding before initiating an infection with rAcMNPV (duplicate flasks).
4. Infect the fed-batch culture at ICDs of 4–10 × $10^6$ cells/mL and MOIs of 5–10 PFU/cell. Maximum volumetric yields can be expected at PCDs of 7–14 × $10^6$ cells/mL, depending on the protein being expressed [62, 65].
5. If not infected, then the fed-batch culture should reach a PCD of approximately 2 × $10^7$ cells/mL at 4–5 days post feeding.
6. If desired, then this SFF (or others like it) may be divided into multiple fractions, with each fraction added at specific time intervals over several days [45, 66]. For example, the feed may be divided into 3 equal volumes, or 3 exponentially increasing volumes, which are added sequentially to the culture at 0, 1, and 2 days after the start of feeding.

### 3.3.7 Cell Density and Cell Viability Enumeration

Cell densities are usually determined via a counting chamber (Improved Neubauer hemocytometer) and a phase-contrast microscope, as follows (also, *see* Chapter 1 for methods of determining cell density and viability):

1. Estimate the dilution required so that approximately 100 cells are counted on each side of the hemocytometer (each side being a counting grid with nine large squares, each being 1 mm in length and 0.1 mm in depth, with a cover slip).
2. Perform 1:1 serial dilutions of the culture sample with medium, but use 0.1 % (w/v) Trypan Blue (prepared in medium) as the diluent for the final dilution.

3. Load each side of the hemocytometer (with cover slip) with 10 μL of the diluted sample, and count the number of cells on each side with a click counter under the microscope (count unstained and stained cells separately).
4. Tally up the number of viable (unstained) and total cells (unstained + stained) from both sides of the hemocytometer (18 squares).
5. Calculate the viable and total cell density using the following formula: cell density (cells/mL) = (no. of cells in 18 squares × dilution factor)/0.0018 mL.
6. If the cell density is estimated from 3× hemocytometer counts (~600 cells counted from 54 large squares), then a relative error of approximately 15 % is obtained [84].

Alternatively, viable and total cell densities may be determined using an automated imaging counting system (e.g., Cedex or ViCell (this can also determine the total cell density)), and total cell densities may be determined using a Coulter Counter (e.g., Multisizer 4), according to the manufacturer's instructions. When using an imaging counting system, the analytical settings optimized for uninfected cells may not work well for infected cells (e.g., underestimated cell viabilities), in which case such settings should be re-optimized.

*3.3.8 Cell Cryopreservation: Freezing*

Insect cells adapted to SFM can be frozen in liquid nitrogen for long-term storage using the following procedure, which works well for Sf-9, HzAM1, and Tn-5 cells in our laboratory. The general principle of animal cell cryopreservation is to freeze slowly and thaw quickly. Insect cells are frozen as follows:

1. Prepare a 100 mL suspension culture, seeded at $5 \times 10^5$ cells/mL in a 250 mL shaker flask.
2. Prepare 15 % (v/v) DMSO in fresh medium in a 10 mL centrifuge tube and store at 4 °C.
3. When the cells are at mid-exponential growth phase (e.g., $2 \times 10^6$ cells/mL, >95 % viability, 24 h doubling time), aliquot 10 mL culture into a 10 mL centrifuge tube, centrifuge at 100 × *g* for 5 min, transfer the supernatant into a new 10 mL tube, and chill on ice (conditioned medium). Also chill the tube of 15 % (v/v) DMSO in fresh medium on ice. Perform **step 3** for ~1 h before conducting **step 5**.
4. Label 1 mL cryogenic vials with the designated freeze numbers.
5. Aliquot the remaining culture (90 mL) into 2 × 50 mL centrifuge tubes, centrifuge at 100 × *g* for 5 min, and discard the supernatant.
6. Resuspend the cell pellet in the required volume (9 mL in 10 mL tube) to obtain a cell density of $2 \times 10^7$ cells/mL (tenfold

concentration), e.g., 4.5 mL ice-cold 15 % (v/v) DMSO in fresh medium and approximately 4.5 mL ice-cold conditioned medium (use the pipette's graduation to estimate the final volume of 9 mL).

7. Aliquot 1 mL cell concentrate into each cryogenic vial, ensuring that the 10 mL tube is well mixed between each dispensing step, and chill on ice.
8. Insert each vial into a short length of Cryoflex™ Tube Wrap, and seal each end of the Cryoflex with the aid of a spirit lamp and scissor clamps.
9. Install the Cryoflex vials in a "Mr Frosty" freezing container filled with isopropyl alcohol, and place in an ultra-low temperature freezer (–80 °C). The "Mr Frosty" container ensures a slowed-down freezing rate of –1 °C/min.
10. On the next day, remove the vials from the "Mr Frosty", install them on appropriate freezing canes, and store them under liquid nitrogen in a cryogenic dewar.

*3.3.9 Cell Cryopreservation: Thawing*

1. Remove a vial from the cryogenic dewar, and place it on dry ice for transport.
2. Rapidly thaw the frozen cells by placing the vial in a 28 °C water bath. Swirl the contents gently to speed up thawing.
3. In a BSC resuspend the thawed cells and transfer into a 10 mL centrifuge tube and add 5 mL fresh SFM.
4. Centrifuge the tube at $100 \times g$ for 5 min, discard the supernatant, and resuspend the cell pellet in fresh SFM to a final volume of 25 mL (~$8 \times 10^5$ cells/mL) in a 125 mL shaker flask (passage 1 after thaw).
5. Incubate the cells and monitor cell growth, viability and sterility daily. Resume the cell passaging routine as described previously (*see* **Note 8**).

## 4 Notes

1. The chemicals and ingredients are usually weighed out in advance for time efficiency, e.g., 1–2 days prior to medium or feed preparation, and stored at room temperature (unless requiring refrigeration).
2. Solution preparation vessels should be covered between ingredient addition and mixing steps to minimize exposure to airborne contaminants. Alternatively, small-scale solution preps may be conducted in a BSC or cross-flow cabinet.
3. The large water bath for lipids emulsification is preferably transparent so that the position of the impeller and vortex formation in the measuring cylinder can be easily adjusted.

4. The 2 L measuring cylinder used for lipids emulsification should have a wide flange at the bottom to facilitate its immobilization in the water bath with a heavy object (we use a 10 kg stainless steel object).
5. If the solution is difficult to filter (significant back pressure), then it may be due to filter blockage (e.g., undissolved particles, protein content) or high viscosity. To minimize particulates, try mixing the solution for a longer period or modify its physico-chemical parameters (e.g., pH). Some ingredients may not be fully dissolvable; if this is the case, then either use multiple filters or choose filters with a prefilter or larger filtration area. For high viscosity solutions (e.g., glucose concentrate) filtration is generally not a problem provided that the flowrate is reduced and the filtration time is increased to avoid a large pressure build-up (causing the filter or tube to rupture).
6. For large-scale filtration it is ideal to have three operators during the procedure, i.e., one to hold the filter capsule and to handle the bottle exchange (BSC), the second to uncap and recap bottles (BSC), and the third to manage the pump and to ensure that the tubing is below the liquid surface (to avoid air being pumped into the filter capsule).
7. Insect cells will obviously exhibit different growth characteristics depending on the medium used. One of the most important initial tasks in insect cell culture is to establish a cell growth curve (cell density and viability over time) for a particular medium, from which the cell doubling time, mid-exponential growth phase and PCD can be determined (*see* Chapter 1). This information is used to set the cell density and temporal parameters for passaging events.
8. Cryopreserved cells should have recovered (i.e., normal cell growth rate and viability) by 2–3 passages after thaw. If not, then discard the cells and thaw out another vial from the cell bank. If there is a low success rate of recovering cells from a particular cryopreserved batch, then the freezing procedure may not have been carried out properly. In this case, repeat the cryopreservation procedure with a new batch of cells.

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# Chapter 9

# Routine Maintenance and Storage of Lepidopteran Insect Cell Lines and Baculoviruses

**Dwight E. Lynn and Robert L. Harrison**

## Abstract

The various methods for maintaining (i.e., subculturing, splitting, or passaging) established lepidopteran cell lines are described. Three procedures are presented that are appropriate for different cell lines dependent upon the growth characteristics (in particular, cell attachment properties) of the cells of interest. In addition to the routine maintenance of cells in active culture, methods are also described for both short-term (low temperature) and long-term (frozen in liquid nitrogen) storage of cell lines, as well as quality control procedures for the cultures. Methods for storing baculoviruses for use in cell cultures and issues of concern when using cell cultures for their production and study are also described.

**Key words** Insect cells, Cell line maintenance, Cell line storage, Liquid nitrogen freezing, Cell line testing, Sterility, Identification, Baculovirus storage

## 1 Introduction

Given that, as described in Chapter 6, more than 320 distinct cell lines have been reported from 65 lepidopteran species, and these cell lines were established by dozens of different researchers, the variability in procedures for handling these cell cultures is almost limitless. Some unique methods have been observed over the years (one postdoc that will remain nameless would throw cultures of a particularly tightly attached cell line across the room to dislodge them for subculturing; the reader may be comforted to hear this individual is no longer a bench scientist), but experience indicates that one of the three methods described here can be adapted to any insect cell line in existence.

The key to successful maintenance of cell cultures is the use of proper aseptic technique. This is something that is more easily taught in person than by written instructions, but effort is made in the procedures described below to point out some specific actions that should be avoided. A few points that bear repeating: attempts at thrift, such as using a pipet for multiple transfers of materials

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(medium or cells) can be a bane to cell culture; work with only *one cell strain* in a hood at a time; use a *different container of medium* for each cell strain; and label cultures with the cell strain designation *before* seeding with cells and compare the name on the parent and daughter flasks during the transfer.

The subculture techniques described here were taken nearly verbatim from a paper written many years ago [1] because few changes have been made in procedures since that publication. In addition to the procedures outlined below, novices to cell culture should also refer to some general cell culture publications [2, 3]. While these publications are intended primarily for researchers working with human and other vertebrate cell types, the basic procedures used with insect cell cultures are not dramatically different. In reality, the common use of phosphate buffered medium for lepidopteran cells greatly simplifies their maintenance when compared to mammalian cultures that use carbonate buffers (which necessitate the use of $CO_2$ incubators) and, as poikilotherms (cold-blooded animals), insects, and their cells can survive, and even thrive, at a much broader range of temperatures. Thus, cultures can be maintained under much less stringent temperature regimens or even without an incubator. Additionally, while a subculture procedure for enzymatic detachment of cells (e.g., trypsinization) that is commonly used with vertebrate cells has been outlined below, this procedure is rarely necessary for the generally much less firmly attached cultured insect cells.

In addition to the techniques for handling cell cultures, quality control procedures are described, including methods for assuring cell identity and testing for contaminants. For cell identity, isozyme and DNA fingerprinting analyses are used, which are adequate for identifying to the species level. The researcher can avoid the most likely contaminants that are an issue in cell cultures (bacteria and fungi) by use of proper aseptic techniques and *not* using antibiotics in the stock cell cultures. Luckily, the less obvious microbes, such as mycoplasmas are less likely to be a problem with lepidopteran cultures because of the lower incubation temperatures, but a method for screening for these agents has been provided in any case.

Finally, methods are discussed for storing cell cultures and issues with virus stocks of which the baculovirologist should be aware.

## 2 Materials

### 2.1 Culturing

1. Mature (late exponential or stationary phase) cell culture.
2. 1, 2, 5, 10, and 25 mL sterile pipets.
3. Insect cell culture medium (appropriate for the cell line of interest) (*see* **Note 1**).

4. Sterile tissue culture flasks (*see* **Note 2**).
5. Sterile 200 μL pipet tips.
6. Trypsin diluent: 800 mg NaCl, 20 mg $KH_2PO_4$, 20 mg KCl, 150 mg $Na_2HPO_4 \times 7H_2O$, 23 mg; $Na_2EDTA$ in demineralized water to 100 mL. Adjust osmotic pressure with 15 % NaCl to 350 mOsm/kg and pH to 7.0 with 2 N NaOH. Filter sterilize through 0.2 μm filter and store at 4 °C.
7. VMF trypsin (virus/mycoplasma free; cell culture tested) (0.05 mg/mL in diluent).
8. Sterile 15 mL centrifuge tubes.
9. 70 % ethanol.
10. 0.4 % Trypan blue solution (optional).

### 2.2 Quality-Control/ Storage

1. Hoechst 33258 fluorescent stain.
2. Agarose electrophoresis gel on plastic backing.
3. PHAB buffer: 50 mM Sodium barbital, 10 mM barbital, 10 mM sodium chloride, 1 mM EDTA in demineralized water (*see* **Note 3**).
4. Malic enzyme substrate solution (ME): 0.27 g DL-Malic acid, 2.18 g M Tris–HCl (pH 7.5), 25 mg $MnCl_2 \cdot 4H_2O$, 15 mg MTT tetrazolium, 15 mg NADP, 10 mg phenazine methosulfate, 30 mL $H_2O$ (*see* **Note 3**).
5. Isocitrate dehydrogenase substrate solution (ICD): 2.18 g Tris–HCl (pH 7.5), 100 mg $MnCl_2 \cdot 4H_2O$, 50 mg $MgCl_2 \cdot 6H_2O$, 50 mg DL-$Na_2$-isocitrate, 15 mg MTT tetrazolium, 15 mg NADP, 10 mg phenazine methosulfate, 30 mL $H_2O$ (*see* **Note 3**).
6. Phosphoglucose isomerase substrate solution (PCI): 2.18 g Tris–HCl (pH 8.0), 76 mg $Na_2EDTA$, 120 mg $MgCl_2 \cdot 6H_2O$, 210 mg α-D-glucose-1-phosphate, 6 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL $H_2O$ (*see* **Note 3**).
7. Phosphoglucomutase substrate solution (PGM): 2.18 g Tris–HCl (pH 8.0), 76 mg $Na_2EDTA$, 120 mg $MgCl_2 \cdot 6H_2O$, 20 mg fructose-6-phosphate, 25 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL $H_2O$ (*see* **Note 3**).
8. Extraction buffer: 10 % (v/v) Triton-X in 0.6 M Tris-EDTA buffer (pH 7.5) in demineralized water.
9. Stabilization buffer: a proprietary material containing sorbitol and Tris–HCl buffer (*see* **Note 3**).
10. Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany): a kit for isolation and purification of DNA.

11. Calcium/magnesium-free phosphate-buffered saline.
12. Ethanol.
13. Custom-synthesized oligonucleotides: available from any oligonucleotide synthesis service, such as Integrated DNA Technologies (IDT, Coralville, IA).
14. illustra™ puReTaq Ready-to-Go PCR Beads: pre-mixed reaction components for performing polymerase chain reaction (PCR) amplifications, from GE Healthcare Life Sciences (Uppsala, Sweden).
15. Molecular biology-grade agarose: for agarose gel electrophoresis.
16. Tris-acetate-EDTA buffer (TAE): 40 mM Tris–HCl base, 20 mM acetic acid, and 1 mM EDTA pH 8.0; a buffer for agarose gel electrophoresis.
17. Ethidium bromide.
18. DNA size standards: a collection of DNA fragments of defined sizes, in the range of 100 base pairs (bp) to ≥5 kilobase pairs (kbp).
19. Glycerol.
20. Dimethylsulfoxide (DMSO), sterilized and cell culture tested.
21. Glass freezing ampoules, capped with aluminum foil and heat sterilized or autoclaved.
22. Long Pasteur pipets, cotton plugged and autoclaved.
23. Aluminum canes for ampule storage.
24. 1 % Methylene red solution in tall graduated cylinder (250 mL size) at 4 °C.

### 2.3 Equipment

1. Biological safety cabinet (preferred) or laminar flow hood.
2. Inverted phase contrast microscope.
3. Mechanical pipetting device (this may be a rubber bulb, a battery-powered self-contained unit, or aquarium-pump type).
4. Refrigerated incubator (26–28 °C; optional second incubator at 16–18 °C).
5. Hemocytometer.
6. Compound light/fluorescent microscope.
7. Electrophoresis power supply (constant 160 V).
8. Horizontal gel electrophoresis unit.
9. Thermal cycler.
10. Gel imaging/documentation system.
11. Liquid nitrogen dewar.
12. Ampoule-sealing apparatus or gas/air torch with glass rod.
13. Controlled rate freezing apparatus.

## 3 Methods

### 3.1 Stock Cell Cultures: Passage Methods

#### 3.1.1 Preparation of Hood, Examination of Culture, and Labeling of Flask(s)

1. Turn on laminar flow hood and wipe down working surface with ~2 mL 70 % ethanol (keep a 100–200-mL squeeze bottle of ethanol next to the hood for this purpose). The hood should be empty at this point. A biological safety cabinet/laminar flow hood is *not* a place to store buffers, pipets or tips, or other supplies. Doing so can disrupt the flow of air through the enclosure, resulting in a contaminated workspace. Additionally, if you use the same hood for work with insect viruses, a good habit would be to use a UV light for disinfecting the hood overnight and handle the stock cultures of cells early in the day prior to any work with viruses. Additionally, only one cell line should be used in the hood at a time and the working surface should be wiped down with ethanol between cultures. Following this last rule will help avoid cross contamination or mislabeling of cell lines.
2. Remove a mature cell culture from incubator and examine it with an inverted microscope fitted with a 10 or 20× phase-contrast objective. The medium in the culture should be fairly clear and cells should be somewhat refractive under the microscope (Fig. 1). Cells that are hard to see with the microscope due to a cloudy appearance to the medium suggest a bacterial contamination. Such cultures should be autoclaved and discarded.
3. Record passage information in a record book. This information should include the date, the "name" of the culture (cell line designation, passage level, culture ID), the amount to be transferred, and the type, amount and specific source of the

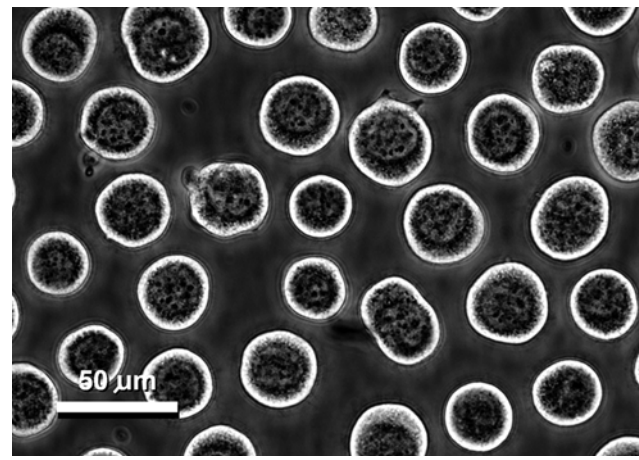


**Fig. 1** *Lymantria dispar* fat body cell line, IPLB-LdFB: An example of an insect cell line that grows in suspension. Phase-contrast microscopy

culture medium to be used (i.e., the individual bottle of medium should be identified in some way; it is recommended that a bottle of medium be marked with the date when it is first opened and that this date be used as the identifier of the bottle). The amount of the old culture to be added to the new (i.e., the split ratio) varies with different cell lines. Some indication of the proper split ratio should be provided by the source of the specific cell line and a range of split ratios should be used during the initial subcultures.

4. Label one or more new 25 $cm^2$ tissue culture flask(s) with the date, cell line designation, and passage number. A fine-tip permanent marker is useful for this purpose.

### *3.1.2 Suspension and Loosely Attached Cell Lines*

This procedure works well for loosely attached and nonattached cell cultures such as *Trichoplusia ni* Tn-368, IAL-TND1, *Lymantria dispar* IPLB-LdFB (Fig. 1), and *Mamestra brassicae* IZD-Mb0503.

1. Place the bottle of fresh medium, the mature culture, and the labeled new culture flask(s) in the hood (*see* **Note 4**). Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5 mL pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything.
2. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. A total volume of 4–5 mL is normally used in a 25 $cm^2$ flask. Therefore, if a total volume of 4 mL is being used with a 1:10 split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see* **Note 5**). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. While a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.
3. Gently swirl the mature culture to evenly disperse the cells. Stand the culture upright and loosen the cap. Using a new, sterile 1 mL pipet, draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the above example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing

fresh medium. Dispense the cell suspension into the flask. Discard the pipet as above.

4. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26–30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70 % ethanol.

### 3.1.3 Moderately Attached Cells

This procedure works well for attached cell cultures such as *Spodoptera frugiperda* IPLB-SF21AE, Sf-9, *L. dispar* IPLB-LdEIta, *Anticarsa gemmatalis* UFL-AG286, *Plodia interpunctella* IAL-PID2, *Plutella xylostella* BCIRL-PxHNU3, *T. ni* BTI-TN5B1-4 (sold commercially as High Five® by) (Tn-5) and IPLB-TN-R$^2$ (Fig. 2), and *Manduca sexta* MRRL-CH1.

1. Place the mature culture in a refrigerator (4 °C) for 20 min.
2. Remove the mature culture and a bottle of fresh medium from the refrigerator and place in the hood with the labeled new culture flask(s) (*see* **Note 4**).
3. Hold the mature culture in one hand and strike it sharply on the side with the palm of the other hand two or three times to loosen the cells. Set it on end in the hood.
4. Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5 mL pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything.

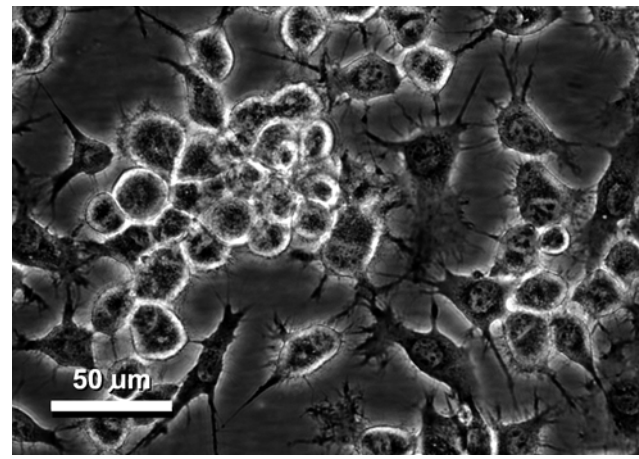


**Fig. 2** *Trichoplusia ni* IPLB-TN-R$^2$ cells: an attached cell line. Phase-contrast microscopy

5. Remove the cap from the medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. If a total volume of 4 mL is being used in a 25 $cm^2$ flask with a 1:10 split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see* **Note 5**). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. While a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.
6. Loosen the cap on the mature culture. Using a new, sterile 1 mL pipet, draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the above example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet as above.
7. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26–30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70 % ethanol.

#### *3.1.4 Strongly Attached Cells: Trypsinization to Dislodge Cells*

This procedure works well for firmly attached cell cultures such as several of the *Heliothis virescens* lines developed in Dr. Lynn's laboratory: IPLB-HvT1, IPLB-HvE1A (Fig. 3), and IPLB-HvE6A.

1. Remove the trypsin solution and the diluent from the refrigerator and place them in the hood with the mature culture and a sterile 15 mL test tube.
2. Remove the cap from the test tube and the mature culture. Take a new, sterile 5 mL pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything. Use the pipet to remove and transfer the medium from the culture to the tube.
3. Loosen the caps on the diluent and trypsin solutions. Remove the cap from the diluent and draw 2 mL of the solution into a new sterile 2 mL pipet. Replace the cap on the diluent and remove the cap from the culture. Slowly release the diluent solution from the pipet letting it wash across the cell monolayer.

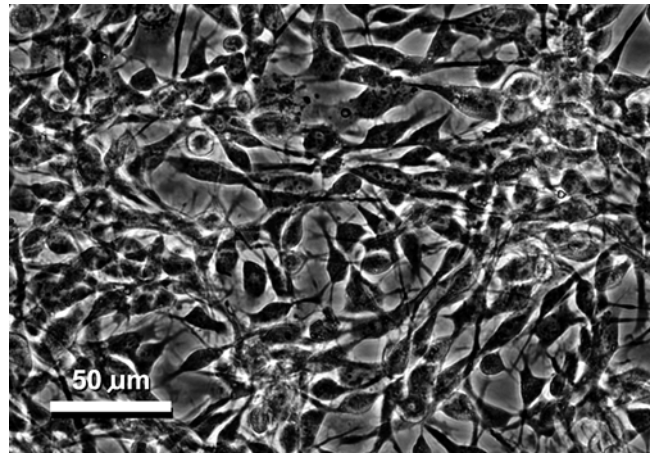


**Fig. 3** *Heliothis virescens* IPLB-HvE1a: firmly attached cells requiring trypsinization. Phase-contrast microscopy

Draw the diluent solution back into the pipet and transfer it to the tube with the old culture medium (eventually this tube and its contents will be discarded). Some cell lines may benefit from a second rinse with diluent.

4. Take a new 1 mL pipet and transfer 1.0 mL from the trypsin solution to the culture. Replace the cap and tilt the culture flask back and forth to ensure that the entire monolayer is wetted by the solution. Set the culture flat on the working surface of the hood and wait 2–5 min. Return the diluent and trypsin solutions to the refrigerator during this waiting period.
5. Tilt the culture once more to ensure that the surface is wet, and then remove the cap and pipet out 0.7 mL of the contents into the test tube with the old medium and rinse solution. Replace and tighten the cap on the culture.
6. Wait 5 more min. Tap the flask gently on the hood and hold the culture up to the light to see if the cells are loosened. It is quite apparent when the monolayer has become detached. If it has not, then tilt the culture again to wet the cell monolayer, lay it flat and wait 5 more min. Repeat this process until the cells are loose.

### *3.1.5 Strongly Attached: Cell Transfer Following Trypsinization*

1. Remove a bottle of fresh medium from the refrigerator and place it and the labeled new culture flask(s) in the hood. Loosen the caps on the medium and new culture flask(s).
2. Take a new 5 mL pipet from the box and open as described above. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. If a total volume of 4 mL is being used in a 25 $cm^2$ flask with a 1:10

split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see* **Note 5**). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. While a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.

3. Loosen the cap on the mature culture. Using a new, sterile 5 mL pipet draw in 5 mL fresh medium and dispense it across the cell surface in the trypsinized culture. Draw the medium back into the pipet and release it a few times to evenly disperse the cell suspension. While some foaming will occur in this process, care should be taken to minimize this since it can result in more damaged cells. Draw in the appropriate amount of the cell suspension (in the above example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet as above.

   *Alternate procedure*: The above procedure assumes that a culture medium containing FBS was used. FBS contains trypsin inhibitors, which stop the activity of the enzyme when the fresh medium is added. If the cells are being maintained in a serum-free medium, then some serum-containing medium (or some other trypsin-inhibiting solution) should be added at this stage. If it is desired to maintain an essentially serum-free culture, then the medium can be replaced in the new flask(s) with fresh serum-free medium after the cells have had a chance to attach (1–2 h after the culture is initiated).

4. Tighten the caps on the medium and old and new cultures and remove them from the hood. Place the new cultures in a 26–30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70 % ethanol.

   *Optional*: Determination of cell viability (for all cell types): After the cells have been suspended into the medium, whether by mechanical or enzymatic means, 0.2 mL of the cell suspension can be mixed with 0.3 mL PBS and 0.5 mL trypan blue (final concentration, 0.2 % w/v) in a small test tube. Place an aliquot on a hemacytometer and count with the compound microscope. The number of viable cells (those not taking up the stain) can be determined and used for initiating the new culture with a precise number of viable cells. However, with experience, one can recognize healthy cells just by examining them in the flask with the inverted microscope.

#### 3.1.6 Backup Cultures

After transferring cells from a mature culture for routine maintenance of a cell line, the left over cells can be left in the original flask and held at a lower temperature (room temperature or lower) as a backup. Adding some fresh medium to the mature culture will assure that the cells can be recovered for two or three weeks. Thus, if a daughter culture is discovered to be unhealthy, then the parent or grandparent culture can be used to recover the cells without needing to resort to using cells from long-term storage.

### 3.2 Quality Control and Testing

The quality of experimental results is directly related to the quality of the cell cultures being used. If the proper technique as described above is employed and the supplies used are obtained from reputable companies, then there is little reason to think that the results will not be valid. Even so, routine testing of cells can help dispel any suspicions of the experimental results. The regularity of such testing will depend on various factors. In a small lab using a single cell strain, the possible cross contamination of cells should be nil, so cell identity should not be an issue. On the other hand, if several cell lines are kept, then it is essential that a rigorous test be employed. Many cell lines have similar morphologies that can change depending on the medium and other culture conditions that are used (Fig. 4).

#### 3.2.1 Sterility

Cell culture medium provides excellent nutrient properties for the growth of many microorganisms and as long as no antibiotics are added to the medium, it usually becomes apparent quite quickly when a culture or medium is contaminated with bacteria or fungi. Simply incubating an aliquot of the medium is an adequate sterility test for microbial contamination in most cases. We typically use 100 mL of a 5 L batch of lab-prepared medium. Sterility checking

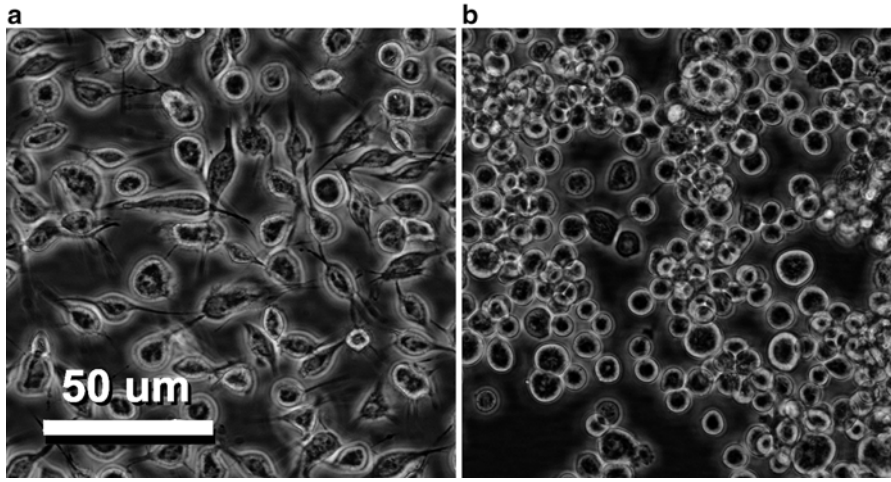


**Fig. 4** *Anticarsa gemmatalis* UFL-Ag286 cells. (**a**) Grown in TC-100 medium, (**b**) In Ex-Cell 400 serum-free medium

of commercially prepared medium does not appear to be necessary. Of course, this requires that you buy your medium from a reputable supplier.

Good cell culture laboratory practice dictates that antibiotics should *not* be used in the cultures used to maintain a cell line. Having an antibiotic in the medium may actually create a greater risk to the cells since a low level microbial contamination could go undetected until antibiotic resistance develops, by which time the problem could have spread to many different cultures being maintained in the laboratory.

Mycoplasma are a potential problem in cell cultures because of their ability to pass through small pore-sized filters (typically used for sterilizing solutions such as culture media that contain heat-labile ingredients) and their indistinct appearance under the microscope. Insect cell culturists are fortunate that the most common mycoplasma species that contaminate cell cultures are associated with mammals (especially human or bovine). These organisms thrive at temperatures typically used for mammalian cells (35–37 °C), but grow quite poorly at temperatures that are used for insect cells. Steiner and McGarrity [4] purposefully contaminated *Drosophila* cell cultures with mycoplasmas and could no longer detect many of these microbes after normal maintenance for a few weeks. Still, a regular testing schedule will assure the researchers that their cultures are clean. One of the simplest assays involves the use of a fluorescent dye, Hoechst 33258, to look for extranuclear DNA as follows:

1. Grow attached cells on glass cover slips.
2. Fix by submersion in 3:1 methanol:acetic acid.
3. Stain with 0.25 μg/mL Hoechst 33258 for 30 min.
4. Mount the cover slips in PBS on a glass slide.
5. Observe with a fluorescent microscope (330–380 nm excitation/440 nm barrier filter). Evidence of extranuclear fluorescence, especially along the cytoplasmic membrane, is suggestive of mycoplasma contamination.

### 3.2.2 Identification: Introduction

Cell line identity is an important issue in cell culture. Nelson-Rees et al. [5] brought this point to light in the 1970s when they revealed that many of the normal human diploid cell lines that were being used in research programs around the world were often not normal (typically cells thought to be from normal human tissues were in fact a line of cervical cancer cells known as HeLa) and, in some cases, were not even human cells. Failure to use the proper cell lines can cause unforeseen responses and negate all the experimental results. Techniques such as chromosome banding patterns were commonly used for identifying mammalian cells but, as a rule, Lepidoptera have rather small chromosomes that become

fragmented easily, making karyotype analysis nearly impossible. Isozyme profiles were determined to be an effective means of identifying cell cultures to the species level [6], as have PCR-based DNA marker methods [7–9]. The methods described here relate to the use of Innovative Chemistry's AuthentiKit™ system to generate isozyme profiles (*see* **Note 3**) and a DNA amplification fingerprinting approach (DAF-PCR) that has been used successfully to detect instances of insect cell line misidentification or contamination [7].

*3.2.3 Identification: Isozymes*

1. Use cells in exponential growth (typically a 5- or 6-day-old culture of cells maintained on a weekly subculture interval).
2. Suspend into the medium using the appropriate method described under Subheading 3.1.
3. Transfer cells to a centrifuge tube and pellet at 1000 × *g* for 10 min.
4. Rinse the cells by resuspending in PBS.
5. Centrifuge at 1000 × *g* for 10 min.
6. Decant and discard the supernatant.
7. Suspend the pellet into an equal volume of extraction buffer (i.e., a volume equal to the size of the pellet. For a nearly confluent insect cell line in a 25 $cm^2$ flask, this is typically between 25 and 100 μL).
8. Place on ice.
9. After 15 min, disrupt the cells by drawing and expelling the cell suspension into a Pasteur pipet 10 times.
10. Centrifuge at 1000 × *g* for 10 min.
11. Mix the supernatant with an equal volume of stabilization buffer. This material can be stored at –20 °C for several months. Apply 1 μL of the cell extract (or a dilution of it, *see* **Note 6**) to a small depression in an agarose gel.
12. Set the gel into the electrophoresis cover (that has been cooled to 4 °C). When fitted onto the base, the two ends of the gel dip into the two buffer chambers containing the positive and negative electrodes, thereby completing the circuit for the electrophoresis.
13. Connect the base to the power supply and run for 25 min.
14. At the end of this time, the gel is laid on a flat surface (with the gel side up/plastic backing down) and 0.5 mL of a specific enzyme (ICD, ME, PGI, or PGM) substrate solution is poured onto the gel and spread evenly across the surface by a single pass of a clean glass rod or 5 mL pipet.
15. Place the gel into a prewarmed incubator tray containing moistened filter paper and incubate at 26–28 °C for 20 min (*see* **Note 7**).

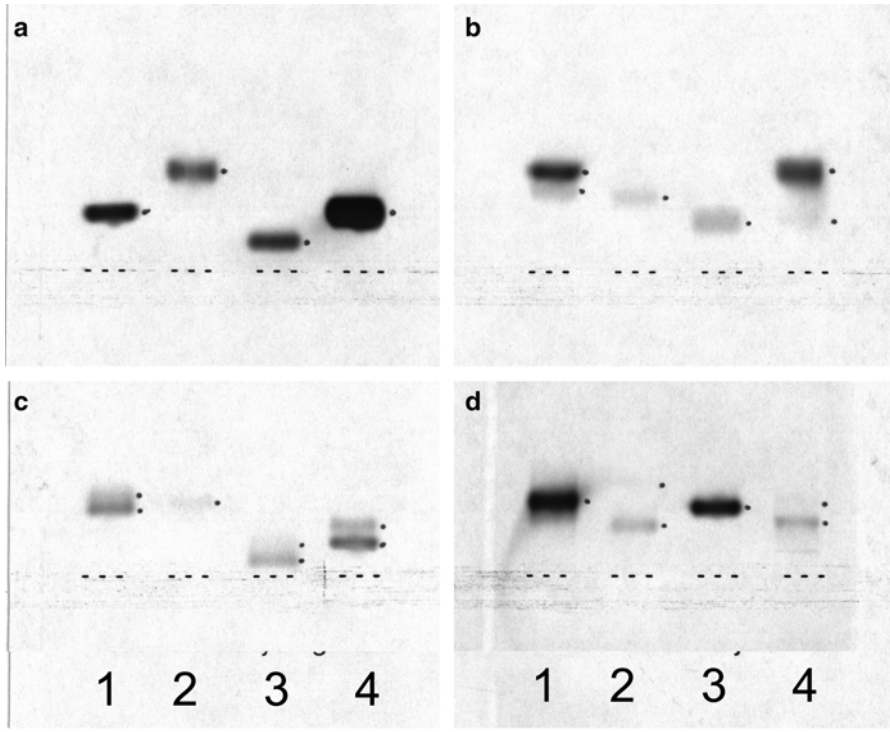


**Fig. 5** Isozyme gels. *Lane 1*, IPLB-Sf21AE cells; *2*, IPLB-CPB2 cells; *3*, IPLB-DU182E cells; *4*, IPLB-PxE1 cells. (**a**) Stained for phosphoglucose isomerase (PCI); (**b**) phosphoglucomutase (PGM); (**c**) isocitrate dehydrogenase (ICD); and (**d**) malic enzyme (ME)

16. Rinse the gel in 500 mL demineralized water with at least one change of the water after about 20 min.
17. Place on a warm surface (under 65 °C) or simply in the flow of air in a laminar flow hood. (On a warm surface, the gel is typically dry in about 30 min, but if room temperature in the hood is used, it may take over an hour.)
18. Once dry, the gel can be placed on a white sheet of paper and the band positions marked with a fine-tip permanent marker and then the location measured from the position of the extract application. The gel itself can be attached to a lab notebook as a permanent record or scanned for use in publications (Fig. 5).

*3.2.4 Identification: DNA Amplification Fingerprinting*

1. Pellet $5 \times 10^6$ cells as above (e.g., as in Subheading 3.2.3, **step 3**), and freeze the pellet at -20 °C. Prior to extraction, resuspend the cells in 200 μL calcium/magnesium-free phosphate buffered saline. For isolating DNA from the insect tissue or developmental stage used to generate the cell lines under study, chill a mortar and pestle in liquid nitrogen for 5 min. Grind approximately 50 mg of the insect or insect tissue. Split the resulting powder between two 1.5 mL microtubes

and re-suspend in a total of 180 μL Buffer ATL (provided with the Qiagen DNeasy Blood & Tissue Kit; *see* **Note 8**).

2. Add 20 μL of proteinase K solution and 200 μL AL buffer from the DNeasy kit to the samples and vortex. Incubate the samples at 70 °C for 10 min.
3. Add 200 μL of ~100 % EtOH and vortex. Transfer the mixture to a DNeasy Mini spin column on top of a 2 mL collection tube. Centrifuge at 6000 × *g* for 1 min. Discard the collection tube and flow-through.
4. Place the column on top of a new 2 mL collection tube. Add 500 μL Buffer AW1 to the column. Centrifuge at 6000 × *g* for 1 min. Discard the collection tube and flow through.
5. Place the column on top of a new 2 mL collection tube. Add 500 μL of Buffer AW2 to the column. Centrifuge at 20,000 × *g* for 3 min. Discard the collection tube and flow-through.
6. Place the column on top of a new 1.5 mL Eppendorf tube. Add 200 μL Buffer AE. Incubate the column at RT for 1 min. Centrifuge at 6000 × *g* for 1 min.
7. Repeat **step 6** and pool the eluate from both elution steps. The eluate contains DNA from the cells or tissues to be used as a template for PCR. Store the DNA sample at 4 °C until use.
8. In preparation for DNA amplification fingerprinting-PCR (DAF-PCR), arrange for the synthesis of oligonucleotide primers to be used in the PC (*see* **Note 9**). When the primers arrive, centrifuge the tubes containing the primers to ensure that the lyophilized oligonucleotides are at the bottom of the tubes. Add DNase-free water to bring the concentration of the primers to 100 μM. Vortex to mix thoroughly.
9. Make a 1:10 dilution of each primer using DNase-free water for a final 10 μM working solution. For each PCR, prepare a primer master mix by adding 2 μL of each of the diluted primers in a pair and 6 μL of DNase-free water, for a final volume of 10 μL primer master mix per reaction. Prepare sufficient primer master mix for one or two additional reactions beyond the number planned for the experiment.
10. Label one tube of illustra™ PURetaq Ready-to-Go PCR Beads (*see* **Note 10**) for each reaction. Prepare one tube for a negative control.
11. Add 14 μL of DNase-free water to each of the PURetaq Ready-to-Go PCR Bead tubes. Adding water to the tube helps dissolve the bead in the tube.
12. Add 1 μL of eluted DNA sample to each tube, and 1 μL water to the negative control tube.
13. Add 10 μL of the primer master mix to the corresponding tubes.

14. Vortex the tubes to mix and microfuge briefly to ensure that the reaction is at the bottom of the tube.
15. Run DAF-PCR on a thermal cycler using the following cycle parameters:
    (a). 95 °C for 2 min.
    (b). 95 °C for 5 s.
    (c). 40 °C for 1 s.
    (d). 72 °C for 30 s.
    (e). Repeat **steps b–d** 39 times.
    (f). 72 °C for 5 min.
16. Make a 2 % agarose gel by dissolving 2 g agarose in 100 mL of TAE buffer in an Erlenmeyer flask. Dissolve the agarose in the buffer by heating in a microwave in short intervals (10–20 s) and mix by swirling between intervals. Once all of the agarose is dissolved, let the agarose solution cool to approximately 60 °C (until the Erlenmeyer flask can be held with a gloved hand). Add ethidium bromide to the gel (final concentration 0.5 μg/mL) and pour into an agarose gel casting tray. Allow the gel to solidify.
17. Load 10 μL of each reaction on the gel along with DNA markers separating reactions set up with different primer sets. Separate PCR products by electrophoresis, following the instructions for the particular electrophoresis unit being used.
18. Take a picture of the gel using a gel documentation system. Compare the mobility pattern of PCR products in the gel between different samples to establish the identity and origin of the cell lines being evaluated (*see* **Note 11**).

### 3.3 Storage

Keeping a stock of cells in some form of storage is insurance against contamination events, deficient media, or change of cultures over time due to selection of cell types inappropriate to experimental needs (*see* **Note 12**). As mentioned in Subheading 3.1.6, keeping cells that are left over from subcultures at a lower temperature as a standard protocol is useful for occasional problems, but other options should be considered.

#### 3.3.1 Low Temperature

Lower incubation temperatures (16–18 °C) can be a useful means of maintaining different cell types in culture. Winstanley and Crook [10] showed this to be an effective means to maintain susceptibility of some cell lines to specific viruses (in their case, a granulovirus). The same methods described in Subheading 3.1 are used for the subculturing. The time interval between splits and the ratios used need to be determined for each cell line of interest, but most insect cell lines can be maintained with a monthly subculture interval and split ratios quite similar to those used when the cells are grown at 26 °C.

### 3.3.2 Liquid Nitrogen

A longer term solution for storage of cells, especially cultures that are not needed on a routine basis, is liquid nitrogen ($LN_2$) storage (*see* **Note 13**). Cells stored in $LN_2$ are very stable, although the freezing and thawing require special consideration since cells are damaged during these processes. Taking our cues from other animal cell cultures, we find a freezing rate of about 1 °C per min causes the least amount of cell damage, while typically a rapid thawing is the best. In addition to the freezing rate, a cryoprotectant is added to the culture medium. The most common of these are glycerol and DMSO. Glycerol is a common substance naturally used by some cold-tolerant insects, suggesting that it would have fewer adverse effects on the cells. However, some cell lines do not survive freezing with glycerol, so DMSO is a viable alternative. DMSO can have toxic contaminants, so a cell-culture-tested supply should be used.

### 3.3.3 Freezing

1. Label the sterilized ampoules with a permanent marker, providing enough information to identify the cells.
2. Using the biosafety cabinet/laminar flow hood, suspend a late, exponential phase culture (typically 5 or 6 days old for cultures kept on a weekly subculture interval) by the normal subculture method (Subheading 3.1). Typically, ten ampoules can be prepared for freezing from a 75 $cm^2$ flask (*see* **Note 14**).
3. Transfer the cells to a 15 mL sterile centrifuge tube and centrifuge at 50–100 × *g* for 5–10 min.
4. While the cells are centrifuging, prepare the freezing medium. This is the normal culture medium (*see* **Note 15**) plus 10 % glycerol (autoclaved) or DMSO (obtain presterilized from most sources of cell culture materials) and should be prepared shortly before use.
5. Decant (and discard) the old medium from the centrifuge tube and resuspend the cell pellet in freezing medium (1 mL per ampoule being prepared—10 mL if using a 75 $cm^2$ flask).
6. Use the sterile, cotton-plugged long Pasteur pipets to transfer 1 mL to each glass freezing ampoule (*see* **Note 16**). Recap with the aluminum foil used during sterilization of the ampoules.
7. Adjust the flame on the sealing apparatus using an empty ampoule of the same type as that used for the cells. Transfer the ampoules to the sealing apparatus a few at a time, removing the aluminum caps shortly before they reach the flame (*see* **Note 17**).
8. Transfer the ampoules from the sealing apparatus to aluminum canes (the end of the canes should be labeled with a permanent marker with the cell line identity) and place them in the methylene red solution in the graduated cylinder for 30 min.
9. Examine and discard any ampoules containing the red dye.

10. Place the remaining ampoules on the canes in the freezing apparatus and start the freezing process (*see* **Note 18**).
11. At the end of the freezing process, transfer the canes to the $LN_2$ dewar.
12. Enter the location of the canes into a log book, including full details on the cell line designation, date of freeze, passage number, culture medium, cryoprotectant, number of ampoules prepared, location in the dewar(s), and person performing the freezing.

#### 3.3.4 Thawing

1. Determine the location of the cell line from the log book.
2. Add 10 mL of the appropriate culture medium (the same formulation used for the cells prior to freezing) to a 15 mL sterile centrifuge tube.
3. Prepare a small beaker (preferably plastic, approximate 150–200 mL size) with water at 30–35 °C.
4. While wearing eye protection (a plastic face shield or goggles), use a pair of forceps to remove the ampoule from the aluminum cane and drop it into the warm water bath (*see* **Notes 13** and **17**).
5. When thawed, wipe the ampoule with a paper towel dampened with 70 % ethanol and, with the ampoule wrapped in the towel, break it at the neck (most currently available ampoules for $LN_2$ freezing are pre-scored for easy breaking. If this is not the case, then use a triangular file to score the glass before breaking).
6. Use a sterile long Pasteur pipet to transfer the contents of the ampoule to the tube of culture medium.
7. Centrifuge at 50–100 × *g* for 5-10 min.
8. Discard the supernatant, resuspend the pellet in 4 mL fresh medium, and transfer to a 25 $cm^2$ tissue culture flask and incubate at 26–28 °C.
9. Examine the culture at 1–3-day intervals and begin regular subculture procedures (Subheading 3.1) once the culture reaches confluency.

### 3.4 Baculoviruses

Hundreds of lepidopteran species have been reported that are infected with baculoviruses [11], although little is known about the majority of these viruses. Most readers of this book are likely to be using the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) that will be provided as a budded virus (BV) sample as part of an expression vector system. These samples can be applied to a susceptible cell line (*see* Chapter 6) and a typical infection (Fig. 6) will be observed within a few days. Much of this book will provide various details about these viruses. The intent with the rest of this chapter is to make the reader aware of some possible issues involved with the use of baculoviruses in continuous cell lines.

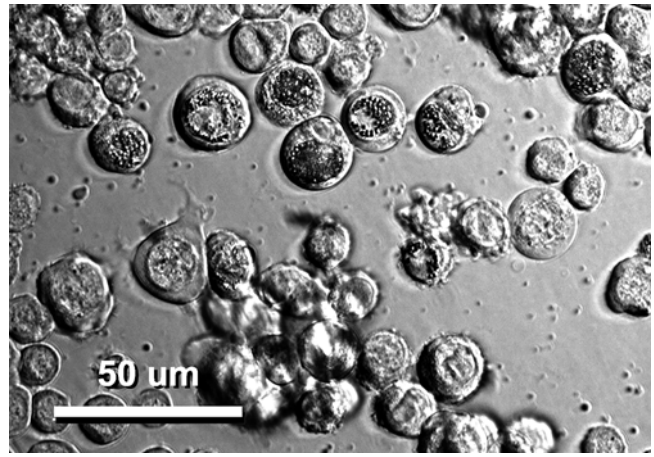


**Fig. 6** IPLB-Sf21AE cell line infected with AcMNPV 72 h post-infection. Differential interference contrast microscopy

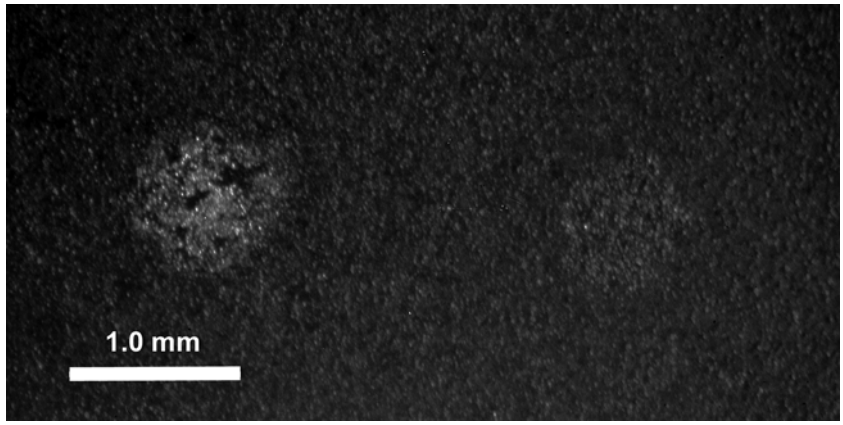


**Fig. 7** MP and FP plaques of AcMNPV in Tn-368 cells stained with neutral red

*3.4.1 Passage Effect*

Two plaque types, named MP (many polyhedra, now called occlusion bodies, OBs) and FP (few polyhedra) (Fig. 7), were described in the first description of a baculovirus plaque assay [12]. Later studies showed that the use of BV progeny over a series of passages in the TN-368 cell line would increase the proportion of FP [13]. *See* Krell [14] for a review of this and other passage effects. The effect can be reversed by feeding OBs to a susceptible insect host [15] or using OB-derived virus (ODV) to infect cell cultures [16]. Cell cultures are much less susceptible to ODV, but this may be an effective alternative to using insects with their potential for having hidden infections with other viruses [17].

In addition to the mutant viruses, defective interfering particles can also be created by multiple passages. The use of low multiplicities of infection is an effective method for avoiding these elements (*see* Chapter 1).

### 3.4.2 Virus Storage

Infectious OBs of baculoviruses are quite stable under a wide range of conditions. Jaques [18] could isolate viable virus from soil after more than 4 years. Lyophilization and storage in airtight containers (even using sealed glass ampoules such as that described above for freezing cells) at refrigeration temperatures in the dark is probably the most stable long-term storage for OB.

Generally, BV is less stable than OB, but can be stored for years in standard tissue culture medium at 4 °C and is even more stable at –85 °C (including stability after exposure to multiple freeze/thaw cycles). Jarvis and Garcia [19] simply placed cell culture supernatant containing AcMNPV directly into the –85 °C freezer without any cryoprotectant addition or any other special procedures. Their medium was supplemented with 10 % FBS; it is not known if similar behavior would be observed with serum-free medium. Furthermore, they found that the most detrimental factor in the storage of BV is light. Thus, care should be taken to use lightproof boxes or to wrap the storage containers (either individual tubes or the boxes) with aluminum foil.

## 4 Notes

1. Grace's insect cell medium was used in the development of the first insect cell lines [20] and is still available from a variety of commercial sources. Grace's medium using Hink's additions [21] are also commonly available. TC-100 (originally described in the literature as BML-TC/10) [22] is a modification of Grace's medium in which a few ingredients subsequently determined to be non-essential or detrimental have been omitted. IPL-41 is a medium developed specifically for IPLB-Sf21AE cells [23] and subsequently formed the basis of many of the commercially available serum-free media for lepidopteran cells, e.g., Ex-Cell 400 series of media (JRH Biologicals), Sf-900II (Invitrogen/GIBCO), and Insect Express (HyClone). While many lepidopteran cell lines will grow in more than one of these media, normal practice is to maintain a specific cell line on the same formulation on which it was originally established until experimental evidence is obtained that the cell line properties of interest (e.g., virus susceptibility) is sustained in an alternate formulation.
2. These come in a variety of sizes, typically measured by the area on the growth surface as 12.5, 25, 75 $cm^2$, etc. sizes. Our own preference is to use 25 $cm^2$ flasks for routine maintenance of cell lines and set up additional cultures in the larger sizes when cells are needed for experiments. Several manufacturers produce tissue supplies, but there appears to be no discernible differences in the growth of lepidopteran cell lines in flasks produced by different manufacturers. In addition to culture

flasks, the same manufacturers produce Petri dishes and multiwell plates. These alternative styles are useful for experiments, but are less desirable for maintenance of cell cultures than culture flasks that can be tightly closed.

3. The gels, buffers, and staining solutions are available as part of Innovative Chemistry's AuthentiKit™. The AuthentiKit is a simple system that uses preformed agarose gels, premeasured buffers, and substrate powders that are dissolved in demineralized water immediately before use. The equipment is also standardized for use in isozyme analysis of cell cultures. While the reader can substitute lab-prepared versions of all these solutions as described in the methods (most of the formulae shown in this paper are from Tabachnick and Knudson [6] or Brewer [24]), the advantage of the AuthentiKit system is the easy reproducibility in laboratories that do not routinely use native gel electrophoresis for protein separations. If lab-prepared materials are being used, then solutions should be prepared fresh each time the isozyme technique is employed. The original AuthentiKit system was designed for mammalian cell cultures and includes seven enzyme substrates. At the same time that this system was being developed, however, Tabachnick and Knudson [6] showed that four enzymes, i.e., malic enzyme (ME), isocitrate dehydrogenase (ICD), phosphoglucose isomerase (PCI), and phosphoglucomutase (PGM), were adequate for identifying a wide variety of insect cell lines to the species level. Innovative Chemistry, Inc. (Marshfield, MA) includes these four enzyme substrates in their catalog, so instead of buying the complete AuthentiKit that includes gels, buffers and substrates for mammalian cell culture identification, one can buy the gels, buffers, and these four substrates as separate items. As an alternative to isozyme analysis, researchers have also used DNA fingerprinting [7] and 2-D gel electrophoresis of cellular proteins [25] for identifying insect cell lines. If you routinely use some of these techniques in your laboratory, then these are valid alternatives for maintaining confidence in your research materials.
4. Some researchers find it beneficial to allow the culture medium to reach room temperature before using it for transferring cells. We have not found this to be an issue for the cultures we maintain and, in fact, we feel keeping the medium cold reduces degradation of components, but the reader should be aware of these different opinions. In the case of the procedure for "strongly attached" cells, since the cells themselves are chilled prior to subculturing, keeping the medium cold is probably advantageous.
5. A small trashcan lined with an autoclave bag can be used for this purpose. When the bag is full, autoclave it and then seal it

in a cardboard box prior to discarding in the trash. Unless you are working with known human or animal pathogens, insect cell cultures are not known to be hazardous. On the other hand, to the general public, tissue culture material can look like medical waste and it is prudent to put them through a decontamination process (such as autoclaving) for the peace of mind of refuse workers.

6. The instructions with the AuthentiKit suggest checking the level of enzymatic activity in the cell extracts prior to performing the electrophoresis. The procedure essentially involves using a spectrometer for determining the activity in the sample, and can be skipped if it is known how much activity can be expected from insect cells for these enzymes. Typically, 1 μL of extract can be loaded per lane for ME, ICD, and PGM and 1 μL of a 1:2 dilution (using the stabilization buffer for the dilution) can be used for PGI gels.
7. This is another departure from the AuthentiKit instructions. They recommend placing the gels at 37 °C. However, their instructions are designed for mammalian cells and this is the normal temperature at which human cells are maintained. The enzyme systems in insects have optimal activity at their typical growth temperatures, 26–28 °C.
8. The Qiagen DNAeasy Blood & Tissue Kit includes ready-made or concentrated proprietary solutions for extraction and purification of DNA. Cells are incubated with a proteinase K solution and Buffer AL, which contains the chaotropic salt guanidinium chloride, for lysis of cells and denaturation and degradation of proteins that bind or degrade DNA. DNA is then bound to spin-columns provided with the kit, and contaminants are washed out of the column with Buffer AW1 (which also contains guanidinium chloride) and Buffer AW2. DNA is then eluted from the column with Buffer AE (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0).
9. The DAF-PCR protocol described here has been developed and used with the following primer sets: aldolase (aldolase-F, 5′-CCGGAGCAGAAGAAGGAGCT-3′; aldolase-R, 5′-CACA TACTGGCAGCGCTTCA-3′), prolactin (prolactin-F, 5′-CT GGGACAGATGGAGGACT-3′; prolactin-R, 5′-CTCAGGT TTTAATCGAATTT-3′), and interleukin-1 beta (interleukin-1B-F, 5′-ATGAGGATGACTTGTTCTTT-3′; interleukin-1B-R, 5′-GAGGTGCTGATGTACCAGTT-3′). DNA templates prepared for DAF-PCR also have been used with inter-simple sequence repeats (ISSR) primers to distinguish among cell lines derived from different species, as well as cell lines derived from different tissues of the same species and between a parental cell line and clonal isolates derived from it [9].

10. The illustra™ puReTaq Ready-To-PCR Beads are beads pre-dispensed into thin-wall PCR tubes that contain all the reagents for PCR, including deoxynucleotides, Taq enzyme, and reaction buffer. The beads are reconstituted with water prior to adding primers and template DNA. This format reduces the number of pipetting steps required to set up PCRs, and thus reduces errors due to mispipetting and reaction-to-reaction variability in the results.
11. The negative control should be clear or have only a few bands. If not something went wrong, e.g., the reactions were mixed up or the water, tubes, or tips were contaminated. The banding patterns in the cell line and the tissue or species of origin will NOT be exactly the same, but the majority of the bands should be the same.
12. Some reports have indicated that cells lose their ability to replicate baculoviruses [26]. This almost certainly is cell line- and/or virus-dependent and may vary by investigator, being dependent of how carefully the cultures are handled. Cells become quite stable in their properties by the time they have been maintained in culture for a year. The process of freezing and thawing cells also apply selection pressure to the cultures (selecting for cells that are more resistant to the freezing procedure or the cryoprotectant being used). The main difference between selection due to freezing and that from long term culture is that the maintenance selection is much more gradual. If cells capable of replicating your virus are lost during the freezing process, then this would become apparent quite quickly after attempting to infect the thawed cells. Unless you are establishing your own cell lines (as described in Chapter 7), the cells you obtain have likely already been through a freeze.
13. While $LN_2$ is a useful tool for storing cell cultures, the reader should be careful in its use. At –186 °C, exposure to $LN_2$ immediately freezes flesh, causing severe "burns." Additionally, the dewar should be kept in a well-ventilated room since nitrogen is continuously being released and can replace the oxygen in an enclosed space. The reader is strongly encouraged to read the material safety data sheet on $LN_2$ for more details about this material.
14. For most cell lines, this represents between one and two million cells and, as long as the recovery from the thawed cells is reasonably good, should be more than the number typically used for initiating a new culture during normal passages. As discussed later, the thawed culture should be examined regularly and subcultured once the cells reach confluency.

15. Cells maintained in serum-free media are more susceptible to damage during freezing. Better results may be obtained with these cultures if 5–10 % FBS is also added to the freezing medium.
16. Researchers may be tempted to use plastic cryogenic tubes, such as those produced by Nunc. Plastic cryovials generally are only intended to be used in the vapor phase of the $LN_2$ dewar. They should only be used to store cells in liquid nitrogen if (a) they possess internal thread screw-cap closures, and (b) they have been hermetically sealed in an outer protective envelope designed for use in liquid nitrogen, such as Nunc CryoFlex™ Tubing, to prevent entry of liquid nitrogen into the vials. The procedure for preparing and freezing cells in cryogenic vials is otherwise identical to the procedure described for storage in glass ampoules.
17. The glass-sealing apparatus cannot be used effectively in a laminar flow hood. This means the ampoules will be exposed to unfiltered air for a period of time. Fortunately, the opening of these ampoules is fairly small and the heat of the sealing flame will destroy any contaminants that land on the stem, but it is recommended to keep the ampoules covered as much as possible until they are sealed. As an alternative to the sealing apparatus, ampoules can be sealed by hand using a gas-air torch. For this procedure, temporarily attach a glass rod to the tip of the ampoule by melting the glass on both. Then heat the ampoule stem about a centimeter lower until the glass melts. With a smooth motion, twist the rod and pull to form the seal. This procedure can be very effective but it is recommended that one practice on empty ampoules. *Use of eye protection is very important when working with sealed glass ampoules.* If the interior is heated after the seal is complete, then the ampoule can explode. This is also a hazard when removing ampoules from $LN_2$ since any that are improperly sealed will contain nitrogen that will expand dramatically when warmed to room temperature or above, creating a shower of glass shrapnel.
18. The controlled-rate freezing apparatus lowers the temperature 1 °C/min. Viable cultures have also been recovered from ampoules that were placed in a small Styrofoam overnight at –80 °C. The ampules are then transferred to the $LN_2$ for storage.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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# Part IV

## Protein Production with Recombinant Baculoviruses

# Chapter 10

# Small-Scale Production of Recombinant Proteins Using the Baculovirus Expression Vector System

**Jian-Ping Yang**

## Abstract

Numerous technological improvements, including progress in vector design, simplification of virus isolation techniques, and advancements in molecular biology and cell culture technologies, have greatly facilitated the use of the baculovirus-insect cell system for routine production of recombinant proteins. This chapter outlines the basic techniques for small-scale protein production using the Baculovirus Expression Vector System (BEVS), including protocols for titer estimation in 96-well plates, expression optimization in 24-well plates, and recombinant protein expression from adherent and suspension cultures in six-well plates and in 50 mL insect cell cultures.

**Key words** Baculovirus expression vector system (BEVS), Recombinant protein expression, End-point dilution assay

## 1 Introduction

The baculovirus expression vector system (BEVS) continues to evolve as a powerful and versatile system enabling the production of high quality recombinant proteins, ranging from membrane-bound proteins to cytosolic enzymes [1]. There are several advantages of the BEVS compared to bacterial expression systems, including improved solubility, incorporation of most mammalian-cell post-translational modifications (e.g., phosphorylation and glycosylation), and retaining biological function for a majority of proteins [2]. When compared to mammalian cell protein expression systems, the BEVS is considerable cheaper, produces higher expression levels (up to 1 g/L of culture), is relatively ease to scale-up, and is safe to use. In addition, considerable effort has been made in recent years to generate transgenic insect cells for producing more authentic mammalian proteins. Furthermore, recent improvements of the BEVS have greatly facilitated the process of obtaining high titer baculovirus stocks, determining virus titers, and optimizing protein expression. The BEVS is highly suitable

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for producing eukaryotic proteins including the production of pharmaceuticals, pesticides, and vaccines for both academic and industrial applications.

Due to the unique properties of proteins, various parameters should be determined empirically when using the BEVS. For example, the optimal cell line and baculovirus transfer vector, and whether a fusion tag is necessary, should be determined experimentally for each recombinant protein of interest. The three most popular insect cell lines used with the BEVS are the *Spodoptera frugiperda* Sf-9 and Sf-21 cell lines, and the *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5, sold as High Five™ by Life Technologies) cell line. These cell lines can be grown in either adherent or suspension culture depending on the specific application and the users' preference, and gene of interest. The High Five™ cell line typically exhibits greater recombinant protein expression levels than either the Sf-9 or Sf-21 cell lines, with particularly higher expression levels of secreted proteins [2]. The cell density at the time of infection and the multiplicity of infection (i.e., infectious virus particles/cell) can also significantly affect protein yield. Optimal seeding densities of Sf-9 cells for small-scale protein production are listed in Table 1. A variety of vector systems are available and the general considerations for choosing and constructing baculovirus vectors are discussed in Chapters 3 and 4. The most commonly used promoter for recombinant protein expression using the BEVS is the very late polyhedrin promoter. A protein can be expressed as either intracellular or secreted, although secreted

**Table 1**
**Approximate seeding densities of Sf-9 cells and volumes for typical culture vessel sizes for optimal infection**

| Type of vessel | Cell density | Final volume (culture medium + added virus |
|---|---|---|
| 96-well plate | $3.0 \times 10^4$ cells/well | 100 µL |
| 24-well plate | $2.0 \times 10^5$ cells/well | 500 µL |
| 12-well plate | $4.0 \times 10^5$ cells/well | 750 µL |
| 6-well plate | $1.0 \times 10^6$ cells/well | 1 mL |
| 60 mm plate | $2.5 \times 10^6$ cells/well | 3 mL |
| T25 flask | $2.0 \times 10^6$ cells/flask | 5 mL |
| T75 flask | $6.0 \times 10^6$ cells/flask | 10 mL |
| T150 flask | $1.2 \times 10^7$ cells/flask | 20 mL |
| Spinners and shake flasks (all) | $2.0 \times 10^6$ to $2.5 \times 10^6$ cells/mL | No more than half of the total volume of the flask |

recombinant proteins are generally preferred since they are easier to purify. The expression of secreted proteins can be achieved by fusing genes with signal peptides such as GP57 or honey-bee melittin, or by using the native signal peptide of the protein being expressed [3]. In addition, adding a tag to a recombinant protein may further simplify its purification. Several affinity and solubility fusion tags, e.g., a poly-histidine tag (His), strep-tag, glutathione s-transferase (GST), and maltose binding protein (MBP), have been fused to the protein of interest. However, it should be determined whether the additional tag affects the protein's biological function. If the desired application necessitates that the tags be removed post-purification, then the cleavability of the tag from the protein and stability of protein after removing the tag should be initially determined on a small-scale.

In this chapter, small-scale recombinant protein expression for cultures ranging from adherent cultures in 96-well plates to shaker or spinner cultures of up to 250 mL is discussed. The major equipment required for small-scale protein expression experiments are relatively simple: an incubator, shaker, inverted light microscope, and sterile working area [4]. Gene expression can vary 100-fold as a result of the intrinsic nature of the protein. The expression level of the same protein can be enhanced several fold by varying signal peptides, host cell types, and the culture conditions. Small-scale systems provide the means to optimize recombinant protein expression levels by rapidly evaluating various cell lines, multiple virus constructs, and growth and infection conditions. Furthermore, there is a reasonable chance that small-scale production can provide sufficient amounts of protein for crystallization, activity assays, antigen production, etc. The general procedures outlined in this chapter include virus infection and titering, protein expression optimization, small-scale protein production, and insect and medium harvest for protein analysis.

## 2 Materials

### 2.1 Cell Culture and Baculovirus

1. *Spodoptera frugiperda* Sf-9 or other cell line (*see* Chapter 6 and **Note 1**).
2. Insect cell culture medium (*see* **Note 2**).
3. High-titer recombination baculovirus stocks (*see* **Note 3**).
4. 27 °C incubator and shaker incubators (90–150 rpm), $CO_2$ is not required and humidification is optional.
5. Inverted light microscope: 4× and 10× objectives.
6. Air-tight bags or containers.
7. Low-speed centrifuge, 4 °C.
8. 0.4 % trypan blue solution.
9. Hematocytometer.

### 2.2 Titering Virus by End-Point Dilution Assay

1. 96-well tissue culture plate: sterile.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Optional but recommended: multichannel pipettors (2–20 μL and 20–200 μL) or repeating pipettor (capable of accurately dispensing 10 μL).
4. Virus stock(s) to be tested.
5. Sterile 1.5-mL microcentrifuge tubes.

### 2.3 Optimization of Infection Parameters in 24-Well Plate

1. 24-well tissue culture plates.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Virus stock(s) to be tested.
4. Optional: Positive control virus (preferable one that expresses a reporter protein, e.g., green fluorescent protein or β-galactosidase).
5. Sterile 1.5-mL microcentrifuge tubes.
6. 4× SDS sample buffer: 0.25 M Tris–HCl (pH 6.8), 40 % Glycerol, 0.04 % (w/v) bromophenol blue, 8 % SDS, add water to 10 mL final volume. Aliquot and freeze at −20 °C until needed. Prior to use, add β-mercaptoethanol (4 % v/v).

### 2.4 Protein Production in Six-Well Plate

1. Six-well tissue culture plates.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Virus stock(s) to be tested.
4. Optional: Positive control virus (*see* Subheading 2.3, **item 4**).
5. Sterile 1.5-mL microcentrifuge tubes.
6. Phosphate buffered Saline (PBS): 1.8 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, 137 mM NaCl, 2.7 mM KCl, adjusted to pH 7.4 with 1 N HCl.
7. 4× SDS sample buffer: The buffer can be diluted to 1× with water and added directly to pellets of insect cells.

### 2.5 Protein Production in 50 mL Suspension Culture

1. 250 mL disposable polycarbonate Erlenmeyer tissue culture flasks.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Virus stock(s) to be tested.
4. Optional: Positive control virus (*see* Subheading 2.3, **item 4**).
5. Sterile 50-mL conical centrifuge tubes.
6. PBS buffer (*see* Subheading 2.4, **item 6**).

### 2.6 Cell Lysis

1. Detergent lysis buffer: 1 % (v/v) Nonidet P-40 (NP40), 15 mM NaCl, 50 mM Tris–Cl, pH 8.0.
2. Protease inhibitors working concentrations (to prevent proteolysis): 1 μM Leupeptin, 0.1 μM Aprotinin, 1 μM Pepstatin A, 1 mM PMSF (phenylmethylsulfonylfluoride).
3. 4× SDS sample buffer: The buffer can be diluted to 1× or 2× with water.
4. Materials for SDS-PAGE and Western blot analysis.

## 3 Methods

### 3.1 Titrating Virus Stocks with the End-Point Dilution Assay

It is important to know the titer of a viral stock when preparing new virus stocks or when infecting insect cells to produce recombinant proteins. The most common approaches for quantifying virus stock are plaque assay and the end-point titration assay. Both of these methods employ cell culture and quantify only the extracellular infectious forms of baculovirus. The detailed protocol for the plaque assay is discussed in Chapter 4; alternative methods of virus titering are discussed in Chapters 4, 11, and 21. Over the years, many different assay systems, including anti-gp64 antibody-based assays, flow cytometry, and real-time PCR-based assays, have been developed to provide rapid virus titrations [5–7], each of which has its own specific strength and weaknesses.

A 96-well plate end-point dilution assay allows many replicates to be performed simultaneously and is technically less difficult than the plaque assay. Various improvements have been described for a rapid end-point dilution assay using reporter proteins such as green fluorescent protein (GFP) [8] or β-galactosidase gene LacZ [9]. However, these methods require additional cloning to modify baculovirus transfer vectors. Here we describe the conventional end-point dilution assay. The basic principle of the method is that a single virus can be replicated and spread throughout the culture, ultimately infecting all cells present and leading to the cytopathic effect. Tenfold serially diluted virus stocks are prepared and added onto replicate monolayer cell cultures in wells of a 96-well plate. After incubating the plates at 27 °C for 7 days, the dilution required to infect one-half of the cultures is estimated by scoring wells based on the cytopathic effect of the virus. The virus titer is calculated as the median tissue culture infectious dose $TCID_{50}$ units/mL and can be theoretically converted to a plaque forming unit per mL (pfu/mL) by the Poisson distribution-derived factor 0.69: $PFU = 0.69 \times TCID_{50}$ [10].

1. Dilute exponentially growing Sf-9 cells with culture medium to a concentration of $2 \times 10^5$ cells/mL. Use a multichannel pipette to dispense 100 μL cell suspension to each well of a 96-well plate and incubate for 1 h allowing cells to attach

firmly. After attachment, cells should appear 25–30 % confluent (*see* **Note 4**).

2. While cells are attaching, prepare tenfold serial virus dilutions. Array 8 sterile 1.5 mL Eppendorf tubes in a rack. Label them as "-2", "-4", "-5", "-6", "-7" "-8", "-9", and "control."
3. Add 990 μL medium to the "-2" and "-4" tubes. Add 900 μL medium to the "-5", "-6", "-7", "-8", "-9", and "control" tubes.
4. Add 10 μL of the virus stock to the tube labeled "-2" (this generates the $10^{-2}$ dilution). Mix by gently vortexing. Mix each dilution well before removing an aliquot for the next dilution to make sure the virus evenly distributed (*see* **Note 5**).
5. Add 10 μL of the "-2" dilution to the tube labeled "-4", mix as above (this is the $10^{-4}$ dilution).
6. Add 100 μL of the "-4" dilution to the "-5" tube and mix well (this is the $10^{-5}$ dilution).
7. Repeat **step 6** for tubes "-6" to "-9". Vortex each mixture (these serial dilutions generate dilutions of $10^{-6}$ through $10^{-9}$).
8. Leave medium only in "control" tube.
9. Add 10 μL of each diluted virus stock to each well in one row of the 96-well plate. That means 12 replicates for each dilution. Move sequentially from the highest dilution to the lowest dilution. Use a new tip for each dilution (*see* **Note 6**).
10. Add 10 μL medium from "control" tube to the last row of the 96-well plate as the uninfected control.
11. Gently mix the virus dilutions in the plate with a circular motion, and then place the plate in a sealed plastic bag containing a moist paper towel (to keep the plate from drying out) and incubate at 27 °C for 7 days (*see* **Note 7**).
12. At the end of the incubation, visually inspect the cells under the microscope and score each well for signs of infection. Wells displaying cytopathic effects are scored with a "+". Cells with cytopathic characteristic have enlarged nuclei, appear much larger than uninfected ones, and often float in the medium. Wells receiving no productive virus particles remain uninfected and are scored with a "–". An example of the scoring results from a 96-well plate end-point dilution is shown in Fig. 1.
13. Count the number of positive (infected) and negative (uninfected) wells for each dilution as given in Fig. 1 and perform the calculation as demonstrated in Table 2. The tissue culture infective dose ($TCID_{50}$) represents the dose that gives rise to cytopathic effects in 50 % of the inoculated cultures. Accumulated values for the total number of virus infected and uninfected wells are obtained by adding in the direction from the lowest to the highest values. The accumulated percentage of virus infected

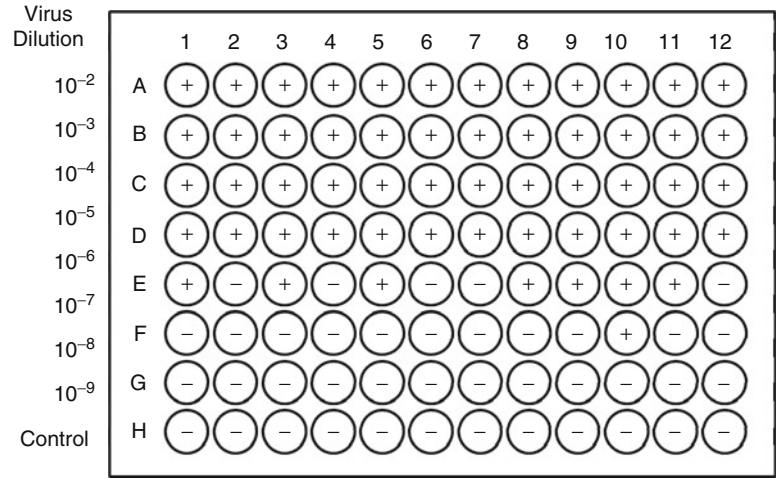


**Fig. 1** An example of results scored with virus infected and uninfected wells by 96-well plate end-point dilution assay

**Table 2**
**A sample spreadsheet using the data from Fig. 1 for calculating the virus titer by an end-point dilution assay**

| | | | Cumulative | | |
|---|---|---|---|---|---|
| **Dilution** | **Infected** | **Uninfected** | **Infected** | **Uninfected** | **% of Infected** |
| $10^{-2}$ | 12 | 0 | | | |
| $10^{-4}$ | 12 | 0 | | | |
| $10^{-5}$ | 12 | 0 | 27 | 0 | **100** |
| $10^{-6}$ | 11 | 1 | 15 | 1 | **93.8** |
| $10^{-7}$ | 3 | 9 | 4 | 10 | **28.6** |
| $10^{-8}$ | 1 | 11 | 1 | 21 | **4.5** |
| $10^{-9}$ | 0 | 12 | 0 | 33 | **0** |
| Proportionate distance (PD) | = (% of wells infected at dilution above 50 % − 50 %) / (% of wells infected at dilution above 50 % − % of wells infected at dilution below 50 %)<br>= [(93.8 − 50) / (93.8 − 28.6)] = **0.67** | | | | |
| $Log_{10}$ ($TCID_{50}$) | = log total dilution above 50 % − (PD × log h) = −6 − (0.67 × 1) = **−6.67**<br>(h is dilution factor. In this case, h = 10) | | | | |
| $TCID_{50}$ | = 10^$Log_{10}$ ($TCID_{50}$) = 10^(−6.67) = **$2.13 \times 10^{-7}$** | | | | |
| $1/TCID_{50}$ | = $1/TCID_{50}$ = $1/(2.0 \times 10^{-7})$ = **$4.70 \times 10^{6}$** | | | | |
| $TCID_{50}$/mL | = $TCID_{50}$/mL per well = $4.70 \times 10^{6} / 0.01$ = **$4.70 \times 10^{8}$**<br>(in this case, 10 μL (0.01 mL) of diluted virus was inoculated into each well) | | | | |
| Pfu/mL | = $0.69 \times TCID_{50}$/mL = $0.69 \times 4.70 \times 10^{8}$ = **$3.24 \times 10^{8}$** pfu/mL | | | | |

cells for each dilution is then calculated as: The accumulated % of infected cells = ((total number of infected wells) / (total number of infected wells + total number of uninfected wells)) × 100 %.

In the example depicted in Table 2, the accumulated percentage of infected cells by $10^{-6}$ dilution is 93.8 %, which is higher than 50 %, and that of the next highest dilution, $10^{-7}$, is 28.6 %, which is lower than 50 %. Thus, the 50 % end-point dilution should lie between these dilutions. The proportionate distance (PD), also known as interpolate value, of the 50 % end-point from these dilutions may be calculated by using the formula of Reed and Muench [11]:

PD = (% of well infected at dilution above 50 % – 50 %) / (% of wells infected at dilution above 50 % – % of wells infected at dilution below 50 %).

The proportionate distance obtained then will be corrected by the dilution factor "h". In this case h (dilution factor) = 10.

$\text{Log}_{10}$ ($\text{TCID}_{50}$) = $\log_{10}$ (total dilution above 50 %) – (PD × $\log_{10}$ (h)).

Because the virus titer is the reciprocal of the $\text{TCID}_{50}$, the value is converted to $1/\text{TCID}_{50}$.

Further, the volume of medium used for infection of wells is taken into account and the titer then is extrapolated to the number of infectious units in 1 mL.

In the example here, because 10 μL (0.01 mL) of infectious medium was added to each well of 96-well plate, so $\text{TCID}_{50}$ / mL = $4.7 \times 10^6$ units / 0.01 mL = $4.7 \times 10^8$/mL.

Finally, $\text{TCID}_{50}$ value can be converted to pfu/mL by the statistical relationship: PFU = 0.69 × $\text{TCID}_{50}$ (*see* Table 2 for step by step calculations).

### 3.2 Optimization of Infection Parameters in 24-Well Plate

Once a high titer virus stock has been obtained, achieving the best expression of a recombinant protein requires the optimization of several parameters. The following protocol can be used to optimize protein expression by evaluating the multiplicity of infection (MOI), host cell line, culture medium, virus construct, and cell density. The method involves growing small-volume cultures of insect cells in a 24-well plate, infecting with virus stock, and periodically withdrawing culture samples that are prepared for protein analysis. The experiment allows determining the kinetic of infection and the optimal harvest time for maximum protein production and minimum protein degradation. The results may provide guidelines for harvesting times in large-scale production systems (*see* **Note 8**).

1. Seed $2 \times 10^5$ healthy exponential phase Sf-9 cells in 200 μL of complete growth medium per well in a 24-well tissue culture plate. Gently tip the plate from side to side four to six times to evenly distribute the cells.

2. Incubate the cells at 27 °C for 1 h to allow cells to fully attach to the bottom of the plate.
3. While cells are incubating, prepare a twofold serial dilution for the virus stock to be optimized. Array five 1.5 mL Eppendorf tubes in an appropriate rack. In the first tube, dilute the virus stock to $8 \times 10^7$ pfu/mL in 500 μL of complete growth medium. Pipette 250 μL complete growth medium into each of the other four tubes. Transfer 250 μL diluted virus stock from the first tube to the next tube and mix well. Transfer 250 μL from tube 2 to tube 3 and mix well. Repeat the dilution for tubes 4 and 5. The MOI from adding 50 μL of each diluted virus stock into each well are equivalent to 20, 10, 5, 2.5, and 1.25 pfu/cell, respectively (*see* **Note 9**).
4. After verifying that the cells have attached to the bottom by inspecting them under an inverted microscope, transfer 50 μL of each diluted virus stock to each of the four wells in one row of the 24-well plate. That corresponds to four replicates per dilution. Leave the cells in the four wells of the last row uninfected.
5. After adding the virus stocks to the plate, gently mix by tilting back and forth, and place the plate in a 27 °C incubator.
6. Harvest the infected cells of one well from each dilution and a control of uninfected cells at various time points (24 h, 48 h, 72 h, and 92 h) post-infection by transferring cells and culture media to centrifugation tubes.
7. Pellet cells by centrifugation 10 min at $1000 \times g$.
8. Prepare sample lysates using the cells if the recombinant protein is intracellular, or supernatants if the recombinant protein is secreted (*see* Subheading 3.5).
9. Analyze protein expression levels by SDS-PAGE or Western blot.

### 3.3 Protein Production in Six-Well Plate

For small-scale procedures, monolayer cultures are preferred for producing viral stocks or recombinant protein because they seem less susceptible to contamination and can more easily be assessed to monitor the progress of viral infections. This basic experimental outline is useful for checking protein expression from new virus constructs.

1. Seed $10^6$ exponential growth phase cells per well in 2 mL of complete growth medium in a 6-well plate. Evenly distribute the cells by gently rocking the plate side to side four to six times.
2. Incubate the cells at 27 °C for 1 h to allow the cells to attach to the bottom of the plate. Verify that the cells have attached by inspection using an inverted microscope.

3. Add the amount of recombinant virus stock to provide a desired MOI in one well (*see* **Note 10**). In a second well, infect with a positive control virus that has previously been characterized at the same MOI. Leave cells in one well uninfected. Use the following formula to calculate the amount of virus stock that should be added to obtain a specific MOI: Inoculum required (mL) = (MOI (pfu/cell) × number of cells) / (titer (pfu/mL) of virus stock). For example, to infect $10^6$ cells using a virus stock with a titer of $5 \times 10^8$ pfu/mL, the volume of virus stock to add to obtain an MOI of 5 is calculated as follows: $(5 \text{ pfu/cell} \times 10^6 \text{ cells})/(5 \times 10^8 \text{ pfu/mL}) = 0.01 \text{ mL} = 10 \mu\text{L}$ virus stock.
4. Gently mix the solution by hand or place on rocking platform for 2 min.
5. Incubate cells at 27 °C in a humidified incubator (or place the plate in a sealed plastic bag with a moistened paper towels to prevent evaporation).
6. At the appropriate time post-infection (previously determined), harvest cells and medium by gently squirting the cells with a sterile pipette to resuspend. Transfer to a 15 mL centrifuge tube and spin gently to pellet the cells. Transfer the cell medium to a fresh tube.
7. For analysis of intracellular proteins, wash the cell pellet twice with cold PBS and resuspend the cells in 1 mL of PBS. Remove a 15 μL sample and add 5 μL of 4× SDS-PAGE buffer. For analysis of secreted proteins, remove a 15 μL sample of the cell medium and add 5 μL of 4× SDS-PAGE buffer (*see* Subheading 3.5).
8. Boil sample for at least 5 min.
9. Process to separate proteins by SDS-PAGE or store samples at −20 °C or −80 °C until needed.

### 3.4 Protein Production in 50 mL Suspension Culture

Most insect cell lines are readily adaptable for growth in suspension, thereby enabling the acquisition of large amounts of recombinant protein. This protocol provides instructions for suspension cell production of recombinant proteins in a small-scale culture. After determining the optimal MOI, time of infection, agitation rate, and temperature for small-scale culture, the culture can be easily scaled up to 1–5 L cultures (for large scale protein production, *see* Chapters 11 and 12).

1. Dilute exponential phase Sf-9 cells to $2 \times 10^6$ cells/mL and add 50 mL of this cell culture to a 250 mL flask (*see* **Note 11**).
2. The desired MOI for protein production is 5–10 (or an optimal MOI previously determined) for suspension culture. The amount of virus needed to add to a culture is calculated as

following: (mL of inoculum needed) = (MOI (pfu/cell)) × (number of cells) / (virus stock titer (pfu/mL)). For example, if the desired MOI is 5 and the virus stock titer is $5 \times 10^8$ pfu/mL, then add 0.4 mL of virus stock to the 50 mL culture at $2 \times 10^6$ cells/mL (50 mL culture × $2 \times 10^6$ cells/mL × 5 pfu/cell) / ($5 \times 10^8$ pfu/mL) = 0.4 mL virus stock.

3. Incubate the infected cells in a 27 °C shaker incubator at 120 rpm (*see* **Note 12**).
4. Harvest cells 72 h post-infection (or optimized harvest time) to 50 mL conical centrifuge tube (*see* **Note 13**).
5. Collect cells by centrifugation for 10 min at 1000 × *g*. Remove medium and transfer to another tube.
6. If the recombinant protein is not secreted, then discard supernatant and wash the cell pellet twice with cold PBS.
7. If the recombinant protein is secreted, then collect the supernatant in a 50 mL high speed centrifuge tube. Remove cell debris by centrifugation at 10,000 × *g* for 10 min. Transfer the supernatant to a new tube.
8. Process sample preparation for protein analysis. The samples can be stored at −20 °C or −80 °C until needed (*see* Subheading 3.5).

### 3.5 Sample Preparation for Protein Analysis

Depending on whether the recombinant protein is secreted, either the cell pellet and/or the supernatant fraction is kept for assessing protein expression levels. The recombinant protein of interest can be detected or quantified either by a biological analysis, a Coomassie blue-stained SDS-PAGE gel, or Western blot analysis (if antibodies against the protein of interest are available). The details of SDS-PAGE, gel staining, and Western blotting will not be discussed here. The lower limits of detection of a protein in the soluble fraction for analysis by Coomassie blue-stained SDS-PAGE and by Western blot are 50 ng and 1 ng, respectively (*see* **Note 14**).

Depending on the protein of interest and the detection method, appropriate methods including detergent lysis, sonication, and freeze-thaw lysis can be used to prepare the lysate. For a cell-associated protein, detergent lysis is a quick and efficient way to lyse cells and release most intracellular and membrane-bound proteins. Protease inhibitors should be added in all cases and the temperature should be kept at 4 °C to minimize protein degradation. Alternatively, insect cells can be directly resuspended in the SDS sample buffer to extract proteins. For a secreted protein, mix the culture supernatant with an equal volume of SDS sample buffer. This provides a quick procedure for preparing lysates for analyzing the secretion level of the recombinant protein (*see* **Note 15**).

Here we describe two basic lysis methods using detergent lysis buffer and SDS sample buffer for protein analysis.

*3.5.1 Detergent Lysis*

1. Place all cell pellets on ice.
2. For cellular samples, add 100 μL of lysis buffer for each $10^6$ cells in the pellet.
3. Add PMSF to each sample to a final concentration of 1 mM (*see* **Note 16**).
4. Vortex each cell sample to break up the cell pellet and incubate all samples on ice for 30–45 min, vortexing at 10 min intervals to assist lysis.
5. After all samples have been lysed, pellet cellular debris by centrifuging at 10,000 × *g* for 10 min at 4 °C.
6. Transfer supernatant (lysate) to a new tube (*see* **Note 17**).
7. Add an equal volume of 2× SDS sample buffer to the lysate.
8. Place the sample tubes in boiling water bath for 5 min.
9. Centrifuge tubes at 15,000 × *g* for 15 min at 4 °C.
10. Load samples directly onto a SDS-PAGE gel or store samples at –20 °C or –80 °C until analyzed.

*3.5.2 Lysis in SDS-PAGE Sample Buffer*

1. For medium (i.e., extracellular) samples, add equal volume of 2× SDS sample buffer to the supernatant. For cell samples, resuspend cells in 1× SDS sample buffer (50–100 μL per $10^6$ cells).
2. Place the sample tubes in boiling water bath for 5 min.
3. Centrifuge tubes at 15,000 × *g* for 15 min at 4 °C.
4. Samples can be loaded directly onto SDS-PAGE gel or store samples at –20 °C or –80 °C until analyzed.

## 4 Notes

1. Sf-9 or Sf-21 cells, but not High Five™ cells, are recommended to generate high-titer virus stocks for high transfection efficiency. Once high-titer virus stocks are generated, Sf-9, Sf-21, and High Five™ cells may be used for protein expression studies. However, the expression level of recombinant proteins may be cell type dependent. It may be necessary to screen a number of cell lines for relative productive capabilities for a given protein. High Five™ cells are particularly well suited for expression of secreted recombinant proteins and provide fivefold to tenfold higher secreted protein expression than Sf-9 cells. At the time of infection, the insect cells should be in exponential growth phase with a viability greater than 95 %. Cell growth can be monitored by cell counting and cell viability with the trypan blue exclusion method using a hematocytometer and a microscope.
2. Various insect cell culture media including serum-containing or serum-free media (SFM) are commercially available and are

suitable for small-scale protein expression. SFM culture may allow cells to grow to a higher density and simplify purification of secreted recombinant proteins. Depending on the application and the protein of interest, it may be necessary to supplement the culture post infection with 0.1–0.5 % FBS or BSA to protect the recombinant protein from proteolysis.

3. Always maintain separate medium bottles for cell culture and for virus work to avoid viral contamination of cell cultures. High-titer stock is approximately $5–10 \times 10^8$ pfu/mL and can be stored in the dark at 4 °C for several months. Do not routinely store used virus stocks at a temperature below 4 °C. Repeated freeze/thaw cycles can result in a 10- to 100-fold decrease in viral titer. It is recommended that serum-free medium be supplemented with 10 % FBS to increase virus stability during long term storage at 4 °C (*see* Chapter 1).
4. The quality of the cell monolayer is critical for a successful assay. The optimal cell density of 20–30 % confluency may avoid rapid nutrient depletion and cell-to-cell contact inhibition. Cells in the early to mid-exponential growth phase gave the highest titer. Be sure to include a cell-only control to assess cell viability, and potential contamination.
5. Vortex virus stocks or dilutions before making the next dilution to ensure that the virus is evenly resuspended. Accurately determining the titer of the virus stock allows for consistency in scale-up expression experiments.
6. We recommend using a multichannel pipettor and changing pipet tips between each dilution to prevent carryover of virus on the tips.
7. A centrifugation step after adding virus particles to cells specifically increases virus adsorption to cells. Twofold to threefold higher titers were observed than in a comparable standard endpoint dilution without centrifugation [12].
8. There are distinct qualitative and quantitative differences between insect cell lines in terms of protein expression levels and the extent of proteolysis. High Five™ cells generally give higher protein expression levels than Sf-9 cells, but proteins expressed in High Five™ cells may undergo more proteolysis. A time course experiment allows for the determination of the expression kinetics for your recombinant protein and the best time to harvest the protein. Maximum expression of secreted and non-secreted proteins are generally observed between 30–72 h and 48–96 h post-infection, respectively. If the protein is toxic to cells, then the cells should be harvested earlier, e.g., 18–24 h post-infection.
9. If working with untitered virus stock, then add different amounts of virus to the culture to establish a dose response for protein expression.

10. Optimal MOI varies depending on culture condition, the protein being expressed, cell lines and the infection kinetics of the virus used. For small scale protein production, it is recommended to infect the cells at an MOI of 5–10 in order to obtain a synchronous infection. This results in most cells in the culture being infected simultaneously and thus producing proteins within the same time frame. However, infecting with a low MOI in suspension culture may increase overall recombinant protein yield.
11. The ratio of culture volume to vessel volume should be less than 50 % in order to ensure good aeration of the culture and to minimize mechanical shear effects.
12. An agitation range of 50–120 rpm is recommended to ensure good mixing and aeration. Growing insect cells in serum-containing media (10–20 % FBS) generally provides adequate protection from cellular shear force in small-scale cultures. Growing cells in Sf-900II SFM or SF-900 III SFM does not require addition of shear force protection. Supplementation with a shear force protectant (e.g., Pluronic F-68) may be required for growing insect cells in medium that lack shear force protection, especially for large-scale culture.
13. The optimal harvesting time varies greatly, depending on the virus construct used and type of protein expressed. For some proteins, it may be a good idea to harvest cells before the expression peak to avoid progressive proteolytic degradation.
14. Samples can be loaded on a SDS-PAGE gel and analyzed by either direct detection or immunodetection. When staining with Coomassie blue or silver, look for a protein band at the expected molecular weight that appears in cells infected with recombinant baculovirus but not in uninfected cells. When using Western blot, negative and positive controls are recommended to distinguish the specific and unspecific protein bands.
15. Usually, a percentage of the protein tagged for secretion will remain cell-bound due to the intrinsic nature of the protein. Thus, it is recommended that both the cell lysate and the supernatant be analyzed to determine the effectiveness of the secretion sequence.
16. One or more protease inhibitors should be added to the lysis buffers. All the protease inhibitor solutions except for PMSF should be stored at –20 °C. PMSF in isopropanol can be stored at room temperature. PMSF is not stable in aqueous solution and therefore must be added to the lysis buffer just prior to use.
17. If the protein being expressed is a membrane-bound protein, then the cell pellet should also be analyzed because the protein may be located in the pellet.

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# Chapter 11

# Recombinant Protein Production in Large-Scale Agitated Bioreactors Using the Baculovirus Expression Vector System

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## Abstract

The production of recombinant proteins using the baculovirus expression vector system (BEVS) in large-scale agitated bioreactors is discussed in this chapter. Detailed methods of the key stages of a batch process, including host cell growth, virus stock amplification and quantification, bioreactor preparation and operation, the infection process, final harvesting, and primary separation steps for recovery of the product are presented. Furthermore, methods involved with advanced on-line monitoring and bioreactor control, which have a significant impact on the overall process success, are briefly discussed.

**Key words** Bioreactor, Large-scale, Recombinant protein expression, Fed-batch, High cell density, Insect cell, Lytic system, Baculovirus, On-line monitoring, Serum-free medium

## 1 Introduction

The BEVS is widely used for the manufacture of biologics, including vaccines for humans and animals, therapeutics, gene therapy vectors, and biopesticides [1]. It is also widely used for small-scale production of proteins for structure-function studies. At its core, this system utilizes a recombinant baculovirus in which a foreign gene is inserted into a nonessential region of its genome and used in conjunction with an insect cell host to produce a recombinant protein. The genes expressed under the very late polyhedron and *p10* promoters are not needed for virus replication; therefore, these sites can be used for foreign gene expression (*see* Chapter 2). It is a versatile system and has the ability to produce a wide variety of proteins such as human kinases, adenosine triphosphate (ATP) binding cassette superfamily proteins, mammalian transferases, chaperones, and higher order proteins and protein complexes such as antibodies, virus-like-particles (*see* Chapter 14), and viral vectors

David W. Murhammer (ed.), *Baculovirus and Insect Cell Expression Protocols*, Methods in Molecular Biology, vol. 1350, DOI 10.1007/978-1-4939-3043-2_11, 

[2]. The commonly used cell lines are *Spodoptera frugiperda* Sf-9 and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5, sold commercially as High Five™ by Life Technologies), but others have been selected or engineered to combine high yield production and the ability to grow to high cell densities (e.g., the commercially available expresSF+ cell line) or to have the capability to produce mammalian like glycosylation [3] (*see* Chapter 18). The expression levels are generally high, with reported yields of 300 mg/L for human collagenase with Tn-5 cells [4] (*see* **Note 1**) and the protein is produced with many posttranslational modifications, usually rendering them biologically active [5].

For most of these applications it is necessary to develop large-scale processes that can operate in an efficient, reproducible, and robust manner. A significant advantage of using insect cells is that they can be easily adapted to grow in suspension culture. The use of media additives, e.g., Pluronic® F-68, a polyol detergent that protects cells against hydrodynamic stress, and supplements such as yeastolate, which has been used in insect cell media to replace serum (*see* Chapter 8), have facilitated the scale-up of the BEVS in agitated bioreactors. Insect cells have been grown in different bioreactor types, which have been operated either in batch or fed-batch modes [6–15]. The use of continuous bioreactor operation for insect cell culture is not common. In our lab, single use wave bioreactors (*see* Chapter 12) have been used as an alternative production system with the BEVS.

In this chapter, the different elements that can influence the final product yield and quality through the insect cell-BEVS process will be discussed. These include the insect cell host, medium, the recombinant baculovirus carrying the gene of interest, cell growth phase at infection, time of infection, multiplicity of infection, nutrient and byproducts concentration in the bioreactor, and bioreactor operating conditions.

## 2 Materials

### 2.1 Cell Culture

1. Host insect cell line(s). A list of potential host insect cells is given in Chapter 6. This chapter focuses on the use of Sf-9 cells in suspension culture using serum-free medium for large-scale production of proteins in bioreactors (*see* **Note 2**).
2. Serum-free medium. Several commercial preparations of serum-free media are available, many of which are discussed in Chapter 8. It is important to note, however, that adaptation of cells to the medium of choice is necessary whenever cells are transferred from one medium to another (*see* **Note 3**).

3. Shake flasks (e.g., 250 mL, 1 L, and 2 L Erlenmeyer flasks with 0.2 μm filter top) for Sf-9 cell growth (*see* Chapter 1 and **Note 4**).
4. Biological Safety Cabinet (Class II).
5. Refrigerated incubator shaker.
6. 0.22 μm filters (Millipore, MA, USA).
7. Inverted light microscope.
8. Hemacytometer and coverslips (Hauser Scientific, Horshaw, PA, USA) (*see* **Note 5**).
9. Two-key manual cell counter (i.e., click counter).
10. Serological pipets: 1, 2, 5, 10, 25, 50 mL single-use presterilized polystyrene.
11. Pipet gun (e.g., Pipet-Aid®, Drummond Scientific).
12. 0.4 % trypan blue solution (e.g., Sigma-Aldrich).

### 2.2 Baculovirus Amplification and Quantification

1. Materials given under Subheading 2.1.
2. Baculovirus stock (*see* **Note 6**).
3. Materials for specific assay.

### 2.3 Cell Growth and Recombinant Protein Production in an Agitated Bioreactor

1. Materials given under Subheadings 2.1 and 2.2.
2. Agitated bioreactor vessel (flat or curved bottom) with height to diameter ratio close to one. A schematic of the bioreactor setup is shown in Fig. 1 [16, 17] (*see* **Notes 7** and **8**).

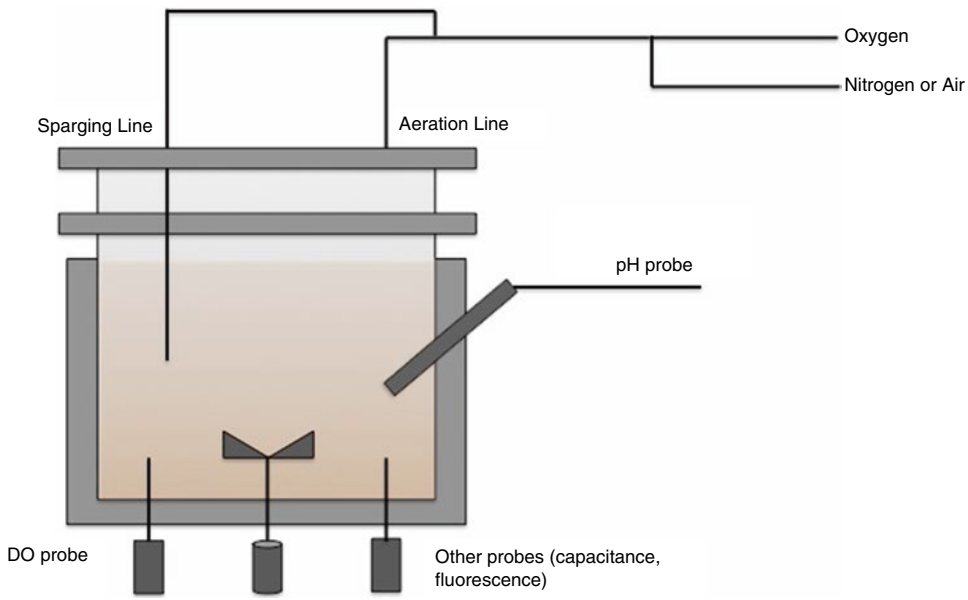


**Fig. 1** Schematic representation of the bioreactor set-up and associated instrumentation

3. Marine, scoping, helical, and/or pitched blade (45° angle) impeller.
4. Sampling port or tube in bioreactor.
5. Sparger in bioreactor (*see* **Note 9**).
6. Agitation speed controller able to control over a wide range of agitation speeds (e.g., 50–300 rpm).
7. Mass flow controllers used to regulate gas flow into the bioreactor (*see* **Note 10**).
8. Temperature probe and jacket temperature controller (water jacket controlled).
9. Dissolved oxygen (DO) polarographic electrode (Ingold, Andover, MA, USA).
10. pH electrode (Ingold, Andover, MA, USA).
11. Additional bottles and tubing for harvest and inoculation.

## 3 Methods

### *3.1 Cell Culture*

#### *3.1.1 Insect Cell Growth for Seeding Bioreactor*

1. Dilute exponential growth phase cells (*see* Chapter 1) with sufficient fresh serum-free medium (preferably pre-warmed to 27 °C, *see* **Note 11**) to obtain a cell density of ~$0.5 \times 10^6$ cells/mL. Determine the cell density of the culture by performing cell counts using a hemacytometer and the trypan blue staining method as described under Subheading 3.1.2 (*see* **Note 12**).
2. Seed Erlenmeyer flasks (*see* **Note 13**) with diluted cell suspension from **step 1**.
3. The volume in the flask should be between 20 and 25 % of the total volume of the flask to ensure adequate oxygen transfer for the growing culture.
4. Maintain cultures in an incubator at 27 °C on a rotating shaker plate at 110–120 rpm (*see* **Note 14**).
5. Continue expanding the cells to additional Erlenmeyer flasks to provide a sufficient number of cells to seed the bioreactor at ~$0.5 \times 10^6$ cells/mL (*see* **Note 15**).

#### *3.1.2 Cell Counting*

The trypan blue staining procedure followed by counting the cells using a hemacytometer is used to obtain viable and total cell counts. The trypan blue method is briefly described below.

1. Withdraw 0.9 mL of sample from each shake flask using a sterile pipette under aseptic conditions and place in a test tube.
2. Immediately return the shake flasks to the incubator.

3. Dilute the sample with growth medium in order to have ~200 cells per side (50 cells for each large square), a range that gives the most accurate cell counts with a hemacytometer.
4. Add 0.1 mL of the 0.4 % trypan blue solution to the 0.9 mL cell suspension (this contributes a factor of 10/9 to the dilution factor D). Mix the solution gently with a pipette. The cell count should be performed about 3–4 min after adding the trypan blue.
5. Withdraw a small volume of the mixed suspension with a pipette, gently place the tip of the pipette on the slot of a clean hemacytometer and allow the suspension to fill the chamber between the slide and the coverslip.
6. Count the number of stained (i.e., dead or nonviable) and unstained (i.e., viable) cells in the four large squares on each corner of the chamber (*see* **Note 5** for the volume of each square).
7. The total and viable cell density can be calculated using Eqs. 1 and 2, respectively:

$$\frac{\text{Total Cells}}{\text{mL}} = \frac{(N + N') \times 10^4 \times \text{D}}{n} \quad (1)$$

$$\frac{\text{Viable Cells}}{\text{mL}} = \frac{N' \times 10^4 \times \text{D}}{n} \quad (2)$$

Where
$N$ = total number of stained (i.e., dead) cells counted
$N'$ = total number of unstained (i.e., viable) cells counted
$n$ = number of large squares in which cells were counted (usually 4)
D = dilution factor (= 10/9 × dilution from **step 3**)

8. The % viability can be calculated using Eq. 3:

$$\%\text{Viability} = \frac{N'}{N + N'} \times 100\%. \quad (3)$$

## 3.2 Baculovirus Amplification and Quantification

Sf-9 insect cells are well suited to the production of baculovirus stocks. *See* Chapter 6 for more details on host cell lines.

### 3.2.1 Virus Stock Amplification

1. Seed Sf-9 cultures in shaker flasks (200–250 mL working volume in a 1-L flask) at ~$0.5 \times 10^6$ cells/mL by diluting an exponential growth phase culture (*see* Chapter 1). Grow the cells in a shaker incubator at 27 °C at 110–120 rpm.
2. Determine the cell density and viability daily (*see* **Note 16**).
3. Add sufficient baculovirus stock to infect the cells at an MOI between 0.01 and 0.1. The cell density at infection should

typically be 2.0–3.0 × $10^6$ cells/mL (*see* Subheading 3.2.2 and **Note 17**).

4. Incubate and determine the cell density and viability daily. It is also useful to monitor the average cell diameter (cell size increases significantly as a result of a baculovirus infection), which is greatly simplified if an automatic cell counter is used (*see* **Note 5**). There is a decline in the viability starting at 48–80 h postinfection (h pi), depending on + suspension at 1000 × *g* and 4 °C for 10 min to remove cells on the MOI used (*see* **Note 18**).
5. Harvest the cells between 72-120 h pi, when the viability is approximately 70%.
6. Centrifuge the cell suspension at 1000 x g and 4°C for 10 min to remove cells.
7. Collect the supernatant and filter immediately through a 0.22 μm filter. Store the viral stock at 4°C until further use for up to 6 months (*see* **Note 19**).
8. Concentrate viral stock if needed.

#### *3.2.2 Baculovirus Quantification*

Several methods can be used to determine the virus titer (e.g., *see* Chapters 4, 5, 10, and 22). These include antibody based assays [18, 19], quantitative real time polymerase chain reaction (Q-RTPCR) methods using baculovirus genome sequences [20, 21], dose-dependent cell growth arrest methods monitored by AlamarBlue [22] or MTT [23], measurement of viable cell-diameter change [24], and baculovirus DNA based labeling methods (*see* **Note 20**). All of the mentioned methods will give similar results, but none will give the exact same measurement. Therefore, it is important to remain consistent with the type of quantification used. It should be emphasized that regardless of the method used to quantify the virus titer, it is important to carry out a few small-scale shake flask experiments to verify the quantity of virus stock needed to achieve growth cessation, which is indicative of a synchronous infection.

### *3.3 Large-Scale Recombinant Protein Production in Bioreactors*

Protein production using insect cells-BEVS can be done in a bioreactor operated in batch, batch replacement or fed-batch modes. In this chapter we will focus on batch mode. Fig. 2 shows the typical growth profile of Sf-9 cells and cell diameter variation during a batch production using an MOI of 1. Viable cell density slightly increases since not all cells are infected by the initial virus addition. Cell diameter starts to increase after infection. In Sf-9 cells, the diameter will usually increase from 14–15 μm to 18 μm during infection. Viability decreases below 90 % by approximately 48 h pi. Fed-batch processes can be considered to maximize yield (*see* **Note 21**) [6, 25].

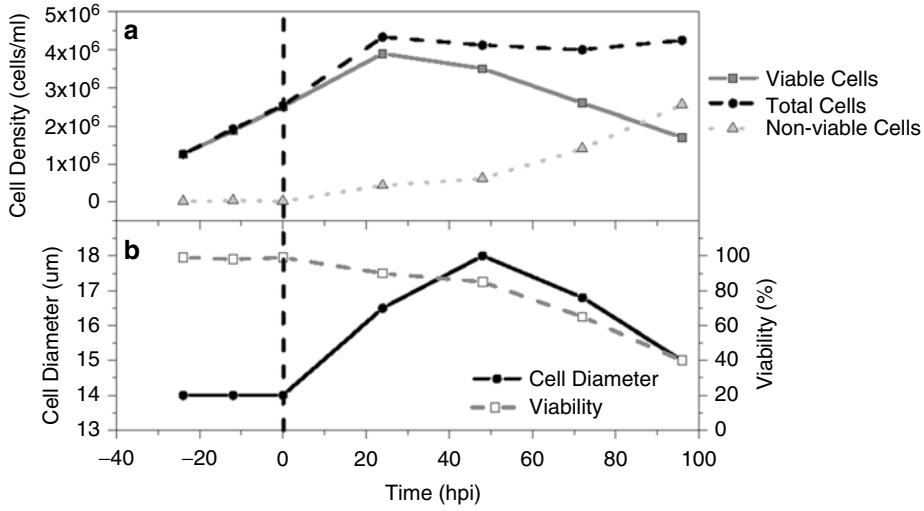


**Fig. 2** (**a**) Typical cell growth and (**b**) diameter profile during protein production at an MOI of 1 in Sf-9 cells

*3.3.1 Bioreactor Sterilization*

1. Clean the vessel and rinse well to remove all traces of detergent.
2. Inspect all material used to keep the reactor sealed (i.e., o-rings); replace as required (*see* **Note 22**).
3. Assemble the reactor following instructions in the operation manual.
4. All accessories, e.g., bottles for adding inoculum, nutrient supplementation, etc., must be prepared prior to sterilization. Autoclavable silicone tubing should be used. The length of tubing attached to inoculum and feed bottles should be based on the distance of the bioreactor from the biological safety cabinets or laminar flow hoods. All sterile transfer is to be done in the cabinet/hood; therefore, it is preferable to locate the bioreactor as close to these units as possible.
5. The pH probe should be calibrated prior to sterilization.
6. The membrane and electrolyte of the dissolved oxygen (DO) probe should be checked and replaced if necessary prior to sterilization. The response time of the DO probe should be checked prior to sterilization. DO probe calibration should be performed after sterilization.
7. Follow the sterilization procedure outlined in the bioreactor manual if available (*see* **Note 23**).
8. Pressure test the reactor for leaks that could lead to contamination (if sterilizing in place, pressure test before sterilization and again when all accessories and probes have been added) (*see* **Note 24**).
9. The sterility of the bioreactor can be verified prior to addition of cells by leaving the medium without cells overnight at 27 °C.

### 3.3.2 Bioreactor Inoculation and Protein Production

Medium, cells, and the virus used for protein production can be added to the bioreactor either by gravity, pumps or pressure (*see* **Note 25**).

1. Inoculate the bioreactor at a cell density of ~$0.5 \times 10^6$ cells/mL by combining cells from exponential growth phase cultures (*see* Chapter 1 and **Note 26**) with fresh medium. It is recommended that the inoculum be prepared in shake flask cultures.
2. The volume of inoculum added should not exceed 10 % of the working volume. This is done to avoid the addition of a large amount of spent medium to the culture at the time of inoculation.
3. Grow the cells to a density of about $2.5–3.0 \times 10^6$ cells/mL, at which point the cells should be infected by adding baculovirus stock (*see* **Note 27**).
4. Synchronous infection may be achieved by using an MOI greater than ~5 (*see* **Note 28**). Since the methods of titration used result in a wide variation of baculovirus stock titers, it is not uncommon to use an MOI of ~10 to obtain a synchronous infection. The volume of baculovirus stock is determined according to Eq. 4.

$$\left\{\begin{array}{c}\text{Volume of baculovirus} \\ \text{stock to add to bioreactor(mL)}\end{array}\right\} = \frac{\mathrm{X} \times \mathrm{V} \times \mathrm{MOI} \times 10^3}{\mathrm{P}} \qquad (4)$$

Where
X = Viable cell density (cells/mL)
V = Bioreactor volume (L)
MOI = Target MOI (pfu/cell)
P = Baculovirus stock titer (pfu/mL)

### 3.3.3 Agitation

The cell suspension needs to be stirred to enhance oxygen uptake for promoting growth and specific productivity. We generally use a setpoint of 110 rpm for a 3.5 L bioreactor, with a pitch blade impeller diameter to tank diameter ($D_i/D_t$) ratio of 0.4–0.6 (*see* **Note 29**).

### 3.3.4 pH

The pH is monitored, but control is usually not required for Sf-9 insect cells (*see* **Note 30**).

### 3.3.5 Dissolved Oxygen (DO)

The specific oxygen uptake rate (OUR) of the culture can be used to monitor the cell's state. In uninfected Sf-9 cells the average specific OUR is 0.22 μmol $O_2/10^6$ cells-h. This respiration rate increases by 30–40 % after infection [26, 27]. To maintain the cell's productivity, the DO inside the bioreactor should be maintained at a set point between 20 and 60 % air saturation (*see* **Note 31**).

#### 3.3.6 Headspace Aeration

To provide oxygen for the cells, headspace aeration with a total gas flow rate of 300 mL/min is sufficient when working at cell densities of $2–3 \times 10^6$ cells/mL at a DO concentration of 40 % air saturation (*see* **Note 32**).

#### 3.3.7 Sparging

When working at higher cell densities (greater than $2–3 \times 10^6$ cells/mL) sparging may be necessary to maintain the DO concentration at the desired set point (*see* **Note 33**).

#### 3.3.8 Harvest

The time of harvest should be determined based on small-scale experiments and is usually conducted when the cell viability drops to between 70 and 90 % (*see* **Note 34**). Generally this occurs between 48 and 120 h pi. Another method to determine when to harvest is based on cell diameter [28]. The typical harvest procedure is given below.

1. Connect a sterile bottle to the harvest line of the bioreactor.
2. Lower the temperature setpoint of the bioreactor to 25 °C or turn off.
3. Decrease the agitation speed to about 60–70 rpm (*see* **Note 35**).
4. Collect the cell culture suspension by opening the harvest valve.
5. The remaining operations may be done at 4 °C or room temperature, depending on protein stability.
6. Centrifuge the cell suspension at $300–350 \times g$. Centrifugation time depends on the size of the culture and centrifugation speed (*see* **Note 36**).
7. Separate the cells and supernatant. Store at –80 °C until further processing if information on degradation rates is not available. If this information is known, then store accordingly.
8. For large volumes of supernatant, it is advisable to carry out a concentration step using ultrafiltration with the appropriate molecular cutoff before storing the product for further purification. Concentration, however, can promote product precipitation and aggregation. Therefore, small-scale experiments should be conducted to evaluate the effect of product concentration to ensure that its quality is maintained during the concentration process.

For intracellular products it may be necessary to first lyse the cells either by mechanical methods or with detergents to release the product before further purification.

#### 3.3.9 Further Advanced On-Line Monitoring and Control of Bioreactor Cultures

Advanced on-line monitoring and control is routinely done to better understand protein production and develop robust processes. The control parameters are DO and pH, although pH is not generally controlled in insect cell culture. Parameters such as carbon

dioxide evolution rate (CER), biomass, and fluorescence (*see* **Notes 31**, **37–39**) can be monitored in addition to DO and pH to gain a better understanding of the process and can be used to optimize accordingly (*see* **Note 40**).

## 4 Notes

1. It should be kept in mind that yields obtained from reporter genes are not always attained on a regular basis for other genes, and yields depend on the type of protein or protein complex produced.
2. Protein Sciences has developed a *Spodoptera frugiperda* cell line, expresSF+, that possesses the ability to grow to high cell densities ($10^8$ cells/mL). This cell line could prove to be very useful for protein production.
3. Serum containing media has the disadvantages of lot-to-lot variability, potential contaminants or adventitious agents and limited availability, to name a few. For these reasons, more groups are using serum-free media (SFM). Most types of lepidopteran cells can be adapted to serum-free media by either the direct or gradual approach (these approaches can also be used when adapting between two different serum-free media). Additionally, baculoviruses produced in different media can be used in a new medium without medium exchange because the addition of no more than 10 % total volume of baculovirus from another medium should not have an effect on the culture. There are several serum-free media that are commercially available that are suitable for insect cell lines from suppliers such as Gibco Invitrogen, Thermoscientific, BD biosciences, Oxford Expression Technologies, Expression Systems and more. New serum-free media become commercially available periodically. *See* Chapter 8 for more details on using serum-free media, e.g., media development and an extensive list of commercially available serum-free media. We use Sf-900 II SFM that comes in dry powder form (*see* Chapter 14, Subheading 2.2). The advantages of using powder medium to liquid are reduced costs, ability to perform extended studies with the same medium batch and long-term storage. Purchased liquid media, however, comes with the advantage of quality control provided by the supplier. Whichever medium is ultimately used, it is advised to filter it with a 0.2 μm sterile filter before use. For example, we generally keep a 10 L 1× concentrated stock at 4 °C that was sterile filtered when it was made. For cell maintenance, we use a 0.5–1.0 L volume of this stock decanted into a new bottle that has been filtered again. Before a bioreactor run, we would use fresh media from the stock that

has been sterilely filtered right before inoculation into the bioreactor.

4. Plastic or glass shake flasks can be used. Wide neck is easier for sampling than skinny. The use of a filter top is advantageous because it reduces the risk of contamination. Tops without a filter need to be slightly left open for gas exchange, which increases the risk of contamination. Generally, the volume of cell suspension in the flask should be between 20 and 25 % of the flask volume (e.g., for cell maintenance we use 125 or 250 mL flasks with 25 or 60 mL cell suspension volumes, respectively). Smaller volumes may result in lower viabilities due to greater shearing of cells during shaking, while higher volumes may lead to oxygen limitations due to inadequate gas/liquid surface area for oxygen transfer. The same principle applies to amplifying cells for bioreactor inoculation. For example, when seeding a 3.5 L bioreactor (2.8 L working volume), cultures of 450 mL in a 2 L flask can be grown to a density of $2 \times 10^6$ cells/mL. These cells can then be used to seed 1.8 L in the bioreactor at $0.5 \times 10^6$ cells/mL. The cell suspension in the bioreactor can be increased to the final 2.8 L by adding fresh filtered medium when cells have grown sufficiently such that the cell density is above $0.5 \times 10^6$ cells/mL following medium addition.
5. The most common hemacytometer design has a volume of 0.1 $mm^3$ (*see* Chapter 1) for each of the large squares (Reichet Brightline, Hausser Bright-Line, Hausser Dark-line). However, some hemacytometers have different volumes (e.g., Ruchs-Rosenthal, 0.2 $mm^3$). Thus, special attention to this detail should be paid since the cell concentration calculation is based on the volume per large square.
6. The problem of virus stock stability may be overcome by storing aliquots of a master virus bank obtained from the early passages (early stages of baculovirus generation after transfection) at −80 °C and if possible in liquid nitrogen (cryoprotectant beneficial effects remain to be studied). The working stock can then be generated from these frozen aliquots. The stability of virus stocks stored at 4 °C can be increased significantly by adding up to 10 % fetal bovine serum [29]. Additional details can be found in Chapter 9.
7. Marine, scoping, and pitched blade impeller types in vessels with height to diameter ratio close to one are more commonly used for animal cells. The low shear requirements for animal cells are achieved by using suitable agitation and aeration conditions (for Sf-9 cells in a 3.5 L bioreactor (2.8 L working volume) with a pitch blade impeller $O_2$ and $N_2$ are supplied at a total rate of 300 mL/min and reactor is mixed at 110 rpm).

Additionally, surfactants, e.g., Pluronic F-68, prevent cell damage resulting from sparging and mixing [30].

8. Bioreactors are available commercially from manufacturers of fermentation equipment. The operation manual for the particular type of bioreactor is an important source of information and outlines most of the procedures to be followed for the physical setup of the equipment. The information on the sterilization procedure for the vessel is also contained in this document. This may vary depending on the system used. Larger bioreactors (with working volumes of 10 L or more) are generally steam sterilized in place. Smaller vessels are often made of glass and are autoclavable. Nonconventional bioreactors, such as the single use wave bioreactor, which is presterilized, can also be used for production (*see* Chapter 12) [16, 17].
9. When taking daily samples, the bioreactor needs to be equipped with a sampling port or tube. If the density is above 2.0–$3.0 \times 10^6$ cells/mL, then the reactor will also need a sparger. Placement of the sparger can be either at the bottom or near the side, but it is important to make sure that it is not placed close to the DO probe to avoid interfering with DO readings. Additional optional probes include capacitance, fluorescence, and infrared detector/outlet gas analyzer.
10. In our lab we use a gas mixture of medical grade $O_2$ and $N_2$. If mass flow controllers are not available, then DO can be controlled through use of sparging and/or mixing. Both sparging and mixing increase the mass transfer rate; therefore, to maintain a setpoint DO these rates can be changed accordingly based on the DO reading. The rate of sparging and size of bubbles may affect cell viability due to shear stress and experiments should be done before at different cell densities to know what sparging and mixing rates can be tolerated [31]. It should be noted that care should be taken when using a gas mixing system designed for mammalian cell cultures that employs $CO_2$, which may result in pH changes during the culture. Insect cells do not require $CO_2$ and accumulation of this gas in the bioreactor could actually be detrimental to the cells and lower productivity. Thus, it is advisable to modify such systems to avoid the use of $CO_2$ altogether by replacing it with $N_2$ or air.
11. Cells can be cultured between 27 and 28 °C. Our lab works at 27 °C.
12. Although the trypan blue method is not a direct measure of viability (it is considered to be a measure of membrane integrity), it is the most commonly used technique to approximate cell viability, especially in the case of infected cells. The application of this protocol is based on the assumption that the

loss of viability precedes the loss of membrane integrity. Alternatively, other cell counting devices, e.g., a CEDEX (Innovatis, Bielefeld, Germany), can be used. The CEDEX provides a means to obtain data on cell parameters (concentration, viability, cell number, and diameter) in a quick and convenient way. It also has a multi-sample function to handle a large number of samples. Another device is the Coulter Counter (Beckman Coulter, Inc., Fullerton, CA) that can determine total cell concentration and average cell diameter. Solokenko et al. [32] studied the relationship between cell size distribution and viable cell population to more accurately calculate biovolumes.

13. The flasks should be cleaned thoroughly to remove traces of detergent prior to steam sterilization. Detergent is used as an agent to lyse cells; thus, if there is leftover detergent in the flasks prior to use, then cell viability and growth are often affected.
14. A refrigerated incubator is generally needed to maintain a temperature of 27 °C. Additionally, the shaker does not need to be humidified to the same extent as shakers used at 37 °C.
15. It is critical that the cells are maintained in the exponential growth phase, i.e., do not allow the cells to grow into the stationary growth phase (*see* Chapter 1). Furthermore, the cells should be healthy as indicated by a viability of 90–99 %. For cell expansion from shake flasks to a 500 L bioreactor, we start with 125 mL flask, followed by a 1 L flask, a 3.5 L bioreactor, a 20 L bioreactor, a 65 L bioreactor, and then the 500 L bioreactor.
16. One sample per day is sufficient for insect cell cultures.
17. The MOI is defined as the ratio of plaque forming units (pfu) to cells at the time of infection. Lower MOIs may also be used for protein production. The MOI should be selected after small-scale testing in shake flasks. The time of harvest may change when the MOI is changed. Additional information can be found in Chapter 1, Subheading 3.2.2.
18. The progress of the infection can be followed by measuring the cell count and size. At these low MOIs there is an initial increase in cell concentration following infection, i.e., resulting from the growth of cells that are not infected by the primary infection. The cell diameter should begin increasing anywhere from 6 to 48 h pi [32]. Harvest should only take place following the concomitant decrease in cell viability and the increase in cell diameter.
19. The filtration of the freshly made virus stock ensures sterility and removes cell debris. The filtration step must be done immediately due to the possibility of a dramatic decrease in

titer following virus stock settling with cell debris (this can occur in a period as short as 1 week). Even with filtering there is sometimes a precipitate that is seen after a week to a month period, which can also decrease the titer. Stocks can be used for many years after production (if frozen); however, their potency and quality will have decreased. For reliable large-scale experiments, a stock of up to 6 months of age can be confidently used. *See* Chapter 9 for more information about baculovirus storage. The titers of virus stocks produced using routine methods are usually in the range of $10^8$–$10^9$ pfu/mL. Additionally, it may be desirable to have higher virus titers when using high MOIs in high cell density cultures, e.g., an MOI of 5–10 is commonly used to achieve a synchronous infection. Baculovirus concentration can be achieved either with ultracentrifugation or by ultrafiltration [33]. This is done to avoid the addition of large volumes of spent medium and the consequent dilution of medium due to large volumes of virus stock needed for high cell density infection. The volume of virus stock added should not exceed 10 % of the total culture volume.

20. The flow cytometry method for baculovirus quantification is based on measurements of labeled virus particles and determines the total virus particle concentration [34]. Specifically, it is based on the binding of a fluorescent dye (SYBR Green I) to the baculovirus DNA. The virus particles are fixed with paraformaldehyde and thereafter permeabilized to allow for the staining of the viral DNA. The number of fluorescent particles, i.e., virus particles, is determined by flow cytometry. This technique directly counts the virus particles, is simple, and can be completed in a matter of hours instead of days that are required for many other titering methods. Another method has been developed by our lab to quantify baculoviruses with HPLC and SYBR green labeling [35]. The baculovirus concentration is determined by using a standard curve developed with different dilutions of well-known concentration standards. This HPLC method is believed to be less time consuming than the flow cytometry method. Both of these methods, however, cannot give direct information on the infectious particles, i.e., they quantify total (infectious and noninfectious) virus particles. It is possible to correlate the total particle number obtained by this method to the pfu/mL obtained using a plaque assay, the end point dilution assay or any other assay that directly determines infectious particle concentration.
21. Fed-batch and batch replacement systems can be used to optimize production. Batch replacement is easy to use and effective for small-scale systems, but becomes uneconomical for large-scale systems. Previous work in the authors' laboratory

showed that a nutrient feed comprised of a full complement of amino acids, yeastolate, vitamins and trace elements [6, 25, 36, 37] results in a 1.4 to 4-fold increase in β-galactosidase volumetric production in a fed-batch Sf-9 cell culture. The work carried out in the authors' laboratory during the early to mid-1990s' on the metabolism combined with subsequent developments in on-line monitoring and control of the bioreactor was used to develop a feeding strategy to achieve very high cell densities of insect cells up to $5.2 \times 10^7$ cells/mL and successfully produce protein at densities of $1.4 \times 10^7$ cells/mL.

22. One leading problem for contamination is an improperly sealed bioreactor. Pressure testing will insure that the reactor is properly sealed.
23. If sterilizing in place, then accessories that need to be autoclaved before sterile attachment (usually in the presence of a flame) to the bioreactor include the inoculation bottle, air filters, sample tube, sample vials, harvest line and bottle, and the capacitance probe (if used). When sterilizing in place, we use the function "heat by water, sterilize by steam" setting for 45 min at 121 °C.
24. To pressure test, introduce air into the reactor up to 15 psi for approximately 15 min. If the pressure drops, then check for leaks at the seals.
25. For small bioreactors (e.g., 3.5 L), it is easier to add medium, cells and virus by gravity with an inoculation bottle. For medium sized reactors (e.g., 20 L), medium and cells can either be added by gravity with an inoculation bottle or pumped at a rate of 200 mL/min. Depending on the volume of virus (no more than 10 % of the working volume), this can be added either with a pump or by gravity. For larger bioreactors (e.g., 65 L), medium and cells are added to the reactor via a line from the previous bioreactor under pressure, or by gravity.
26. We observe a slight lag phase after seeding the bioreactor. If seeded at $0.5 \times 10^6$ cells/mL, then after 24 h the cells are usually at $0.8–0.95 \times 10^6$ cells/mL.
27. Cells are not infected at a density greater than $2.5 \times 10^6$ cells/mL in batch mode because of the cell density effect, i.e., nutrient depletion after baculovirus infection. At higher densities, the gains achieved by infecting a larger number of cells are counterbalanced by the lower specific productivity of the cells. This can be overcome by implementing a feeding strategy to keep specific productivity high while maintaining higher cell densities [6, 38].
28. The MOI is defined as the number of plaque forming units (PFU) per cell at the time of infection. At a given time the

probability of a cell being infected can be approximated by a Poisson distribution [39]. According to the Poisson distribution, the proportion of uninfected cells in a population is given by exp[-MOI]; thus, an MOI of 1 will result in 36 % of the cells remaining uninfected [33]. *See* Chapter 1 for more details about the Poisson distribution. It is also known that cell infection occurs best when the cells are in the exponential growth phase. Therefore, the growth phase of the cells at infection will also play a role in the proportion of cells infected following the addition of virus to the culture. It is recommended that shake flask experiments be used to determine the MOI required to stop growth at early times postinfection, which would be indicative of a synchronous infection. At lower MOI, secondary infection will be required to complete the infection process; as a consequence the production process will be delayed.

29. When controlling the DO with mass flow controllers, agitation should be held constant since changing the agitation rate will change the $O_2$ transfer rate (OTR) into the culture by altering the volumetric oxygen transfer coefficient, $k_La$ ($OTR = k_La(C^* - C_L)$, where $C^*$ is the saturated DO concentration, i.e., $O_2$ solubility, and $C_L$ is the actual DO concentration in the culture). If mass flow controllers are not available, then increasing the stirring speed can promote increased oxygen transfer to help maintain the DO at a desired setpoint. Although most media contain Pluronic F-68 to reduce shear sensitivity, increasing the agitation rate will increase shear on the cells; thus, a gentle balance must be achieved. We recommend DO control using metered gases and sparging versus increased agitation rate.
30. Sf-9 cells do not normally produce significant amounts of either lactate or ammonia as metabolic byproducts [40], which are the main cause of pH change in animal cell cultures. Thus, a significant change in the pH of Sf-9 cell cultures is usually indicative of problems either with contamination and/or lack of oxygen. Oxygen deficiency results in lactate production due to glucose metabolism under anaerobic conditions. In contrast, Tn-5 cells used at high cell densities can result in significant pH change due to byproduct accumulation even with sufficient oxygen levels. However, pH control of Tn-5 cell cultures is usually unnecessary since most of the corresponding pH change only occurs upon cell death. An exception would be if the recombinant protein was particularly pH sensitive and secreted into the medium.
31. In order to maintain the DO, oxygen is fed into the reactor in a gas stream (e.g., air, a $N_2/O_2$ mixture or pure $O_2$), either through sparging and/or headspace aeration, while agitating the medium. In a more advanced system, the composition and

gas flow rate (ratio of $O_2$ to $N_2$) can be controlled with mass flow controllers (MFCs) in order to maintain a DO setpoint. In our lab, we keep the total gas flow rate constant but allow the ratio of gases to fluctuate (all while keeping the agitation rate constant). The composition of the gas then depends on the DO concentration in the bioreactor and the cells' oxygen demand. We monitor this composition using the percent oxygen in the feed. Some providers of MFCs are Brooke Instruments (Hatfield, PA), MKS instruments (Andover, MA) and OMEGA Engineering (Laval, QC) (the MFC from OMEGA are more economical, but high degrees of accuracy may be sacrificed when compared to more expensive MFCs). If mass flow controllers are not available, then total gas flow rates, sparging and agitation rates can be altered to maintain the DO concentration.

32. For a 3.5 L bioreactor the working volume is 2.8–2.9 L, thereby giving a headspace volume of 600–700 mL. For a 20 L bioreactor the working volume is 18 L, leaving a headspace volume of 2000 mL.
33. When using a system with mass controllers, the first indication other than cell density that sparging is needed is when the $O_2$ content in the gas stream reaches 85–90 %. Sparging can be performed continuously or with pulses. In both cases, stirring and headspace aeration remain the same as without sparging. In the case of continuous sparging, the composition of the gas stream should be the same as the stream providing headspace aeration (e.g., 60 % $O_2$, 40 % $N_2$) at a rate of 10 mL/min and continued for the duration of the run. With pulse sparging, pure $O_2$ is used and pulses can start at a rate of 10 mL/min for only about 1 s or less. As the density becomes higher, pulses may need to be conducted for longer periods of time. Care must be taken that the sparger is not inserted too close to the impellers and away from the DO probe.
34. If viability is too low (<70 %), then there may be product degradation from the release of intracellular proteases, and if it is too high (>90 %), then the protein may not be expressed to its optimal level. It should be kept in mind that cell viability is not a measure of cell lysis itself, but an indication that it is occurring. If the viability is too low during extracellular protein production, then the product can be degraded by proteases in the supernatant. When producing intracellular proteins, cell lysis may release proteins into the supernatant, but they may not be properly processed, folded and functional, and there is the risk of degradation from released proteases.
35. Turning the temperature control very close to room temperature or off ensures that the culture cannot heat up, thereby

reducing the risk for protease activity that can degrade the product. Additionally, the agitation is decreased mainly because the cells no longer need oxygenation since they are being harvested and only require agitation to maintain the cells in suspension. When the volume decreases during harvest and agitation is still running at the same speed (i.e., 110 rpm) the shear stress could potentially lyse the cells due to their increased fragility at this time. Harvest could be done without these steps, but is routinely done in our lab as a precautionary measure.

36. When the product is intracellular, lower speeds are used as a precaution to make sure that the cells remain intact since they are fragile at the end of the culture.

37. Insect cells do not require $CO_2$ in the gas mixture. However, the measurement of $CO_2$ in the bioreactor exhaust gas can be used to quantify the cell respiration rate and thus characterize the physiological state of the cells. $CO_2$ can be measured online by passing the vent gas through an infrared analyzer (e.g., Servomex 1400, Norwood, MA, USA) that measures the partial pressure of $CO_2$ in the outlet gas stream. This can be related back to the amount of $CO_2$ dissolved in the culture. The carbon dioxide evolution rate (CER) is defined as the rate at which $CO_2$ is produced by the cells through respiration. The CER can be calculated by combining mass balances of $CO_2$ in the liquid and gas phases and Henry's Law, and by assuming that the mass transfer coefficient and Henry's law constant for $CO_2$ are constant and that there is no $CO_2$ in the inlet streams. It has been reported that there is a surge in the metabolism following infection that is reflected by a peak in the CER, which generally occurs between 18 and 24 h pi [26]. This characteristic has been used in our laboratory to follow the progress of infection in the bioreactor. The CER profile also gives valuable information on whether the infection was synchronous or asynchronous. A delayed peak in the CER indicates that infectivity was insufficient and a nonsynchronous infection has occurred.

38. The details of measuring viable biomass and biovolume using capacitance measurements have been described previously [41, 42]. The measurement principle is based on the charge separation properties of the cell membrane that results in each cell acting as a capacitor. The capacitance of the cell suspension can thus be measured and used to determine the total biomass of the culture. The advantage of using this system is that charge separation can occur only when there is cell membrane integrity. Therefore, dead cells do not act as a capacitor since they cannot maintain their membrane integrity; thus, this method measures only viable cells in the culture. The resulting capaci-

tance profile follows the viable cell density profile closely until the time of infection. Upon infection, there is no significant increase in the cell density (total and viable), but a further increase is seen in the capacitance profile, which can be attributed to the increase in cell diameter. The capacitance profile then closely follows the cell diameter profile. Furthermore, it can be seen that once the cell diameter reaches a maximum value, there is no further increase in the capacitance value, and subsequently shows a decrease concomitant with the decrease in cell diameter and viable cell density. Relative capacitance was shown to be a reliable indicator for cultures growing and infected at low densities [41] as well as for high cell densities [15, 42]. The major vendors of capacitance probes are Aber Instruments (United Kingdom), Applikon (The Netherlands), and Fogale Nanotech (France). They are available in single and multifrequency units. For Sf-9 cell cultures, with a single frequency probe, a frequency of 0.6 MHz is used [41]. For multiple frequency units, *see* refs. [43] and [44].

39. A fluorescence probe could also be used. This would be useful when working with a system that contains a reporter gene, e.g., GFP, to optimize harvest, process conditions and choice of promoter. Additionally, many metabolites are autofluorescent and could be monitored online using multiwavelength probes, which could be a benefit when developing a production feeding strategy. It should be noted, however, that the interpretation of metabolite levels is nontrivial [45, 46]. DELTA (Denmark) is one supplier of fluorescent probes.

40. The combination of the CER profile and the capacitance profile from the biomass monitor can help in designing a nutrient feeding strategy to achieve higher densities and also in making decisions on the time of harvest based on the synchronicity of the infection. The peak in the CER occurs between 18 and 24 h pi in cultures infected at an MOI resulting in synchronous infection. This is accompanied by a shoulder in the profile of the permittivity that shows a plateau, thereby indicating a synchronous infection. In cases where a low MOI is used, i.e., an asynchronous infection, the CER peak shifts to a later time point and the profile of the permittivity continues to increase, thereby showing postinfection growth [26]. It should be noted that following infection there is a competition for available nutrient resources between virus replication, protein production, cell growth, and cell maintenance. Thus, postinfection cell growth observed by the on-line signals of CER and permittivity indicate that additional nutrients may be required. Hence, off-line monitoring of glutamine should be done to ensure that the feeding strategy eliminates nutrient limitations [42].

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# Chapter 12

# Protein Expression in Insect and Mammalian Cells Using Baculoviruses in Wave Bioreactors

**Sue H. Kadwell and Laurie K. Overton**

## Abstract

Many types of disposable bioreactors for protein expression in insect and mammalian cells are now available. They differ in design, capacity, and sensor options, with many selections available for either rocking platform, orbitally shaken, pneumatically mixed, or stirred-tank bioreactors lined with an integral disposable bag (Shukla and Gottschalk, Trends Biotechnol 31(3):147–154, 2013). WAVE Bioreactors™ were among the first disposable systems to be developed (Singh, Cytotechnology 30:149–158, 1999). Since their commercialization in 1999, Wave Bioreactors have become routinely used in many laboratories due to their ease of operation, limited utility requirements, and protein expression levels comparability to traditional stirred-tank bioreactors. Wave Bioreactors are designed to use a presterilized Cellbag™, which is attached to a rocking platform and inflated with filtered air provided by the bioreactor unit. The Cellbag can be filled with medium and cells and maintained at a set temperature. The rocking motion, which is adjusted through angle and rock speed settings, provides mixing of oxygen (and $CO_2$, which is used to control pH in mammalian cell cultures) from the headspace created in the inflated Cellbag with the cell culture medium and cells. This rocking motion can be adjusted to prevent cell shear damage. Dissolved oxygen and pH can be monitored during scale-up, and samples can be easily removed to monitor other parameters. Insect and mammalian cells grow very well in Wave Bioreactors (Shukla and Gottschalk, Trends Biotechnol 31(3):147–154, 2013). Combining Wave Bioreactor cell growth capabilities with recombinant baculoviruses engineered for insect or mammalian cell expression has proven to be a powerful tool for rapid production of a wide range of proteins.

**Key words** Wave Bioreactors™, Cellbag™, Sf-9 cells, CHO suspension cells, Recombinant baculovirus, BacMam, Transduction, Mass spectrometer, SCADA

## 1 Introduction

Wave Bioreactors have gained widespread acceptance since their introduction in the 1990s, and many different applications have been derived over the years. (Other options are now available for rocking platform bioreactors with disposable bags including Biostat RM (Sartorius Stedim, www.sartorius.com/), AppliFlex (www.applikon-bio.com/), Tsunami Bioreactor (CatchMabs BV, PO Box 134, 6700AC Wageningen, Netherlands), and CELL-tainer

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(www.celltainer.com/)) [1]. These applications have included routine laboratory cell culture processes, e.g., scale-up of mammalian protein production cell lines (e.g., CHO, HEK293, NS0, and hybridoma lines) [2], mammalian seed train production for large-scale bioreactor inoculation [3], and insect cell culture (e.g., *Spodoptera frugiperda* Sf-9 [4], *Trichoplusia ni* BTI-Tn-5B1-4 [known commercially as High Five™] [5] and *Drosophila* Schneider 2 [S2] [6] cell lines). The use of Wave Bioreactors has also been expanded to specialized applications, e.g., clinical use for individual patient cell transfer therapy under GMP conditions [7, 8], production of clinical-grade viral vectors [9], plant cell culture [10] and culture of unique organisms, including parasites, as recently reported for the *Plasmodium falciparum* malaria parasite [11]. The high-oxygen demand required to grow *E. coli* has limited their use in Wave Bioreactors, but they have been used to produce inoculum cultures for production fermentations [12]. Wave Bioreactors are attractive for this wide range of applications due to many factors, including those listed in Table 1. For many bioprocessing laboratories, the advantages of disposable systems such as Wave Bioreactors far outweigh the reported disadvantages [13].

Our GSK facility has successfully used baculovirus-infected insect cells for protein production to support early research programs for over 25 years. We have used Wave Bioreactors for over 10 years as many laboratories have replaced glass and clean in place (CIP)/steam in place (SIP) stirred-tank bioreactors with disposable systems. For our processes, the flexibility provided from ease of set-up and operation, fast turnaround time, and proven expression comparability to our traditional methods have actually improved our capabilities and capacity. Suspension insect cell culture requires only temperature control, agitation, and an air supply, thereby making Wave Bioreactors an excellent choice for cell growth.

Simplicity of culture conditions is not, however, a requirement for use of Wave Bioreactors as proven by many of the examples referenced above. Dissolved oxygen and pH probes can be inserted into the Cellbags, sample lines can be connected to automate biochemical analyzers, and exit gas can be analyzed using mass spectrometry (Fig. 1a). Unit operation can be controlled, with data monitored and documented using Supervisory Control and Data Acquisition Systems (SCADA) (Fig. 1b), thereby providing data integrity for use in GMP/GLP manufacturing environments, as well as for routine research laboratory notebook documentation (Fig. 2).

The use of BacMam viruses (*see* Chapter 5) for transient protein expression in mammalian cells is our system of choice to provide proteins requiring complex, functional posttranslational modifications. BacMam viruses provide an inexpensive gene delivery method that does not require transfection reagents or

**Table 1**
**Advantages and disadvantages of wave bioreactor**

| | Advantages | Disadvantages |
|---|---|---|
| Cell line choice | Insect and mammalian | |
| House system requirements | No steam requirements, only electricity required. Any additional gas ($CO_2$, for example) can be supplied from tanks | |
| Bioreactor set-up | Limited training required.<br>Minimizes risk of cross-contamination | |
| Media fill, cell addition, and virus addition | Easily accomplished with high degree of sterility using custom or off-the-shelf Wavebags, addition and media bags if a tube welder is used. Without tube welder, can use quick-connect ends | Purchase of tube welder and sealer recommended.<br>Reports of leachables and extractables from bag materials |
| Sampling | Quick and easy. No need to steam across sample connector. Can also set up "in-line" methods for automatic sampling, including mass spectrometer monitoring. SCADA system can be used for documenting data | |
| Harvest | Similar to other bioreactors | |
| Clean-up | Rapid clean-up: autoclave used bag and discard, or dispose of as biohazardous waste | |
| Yield | Comparable to other bioreactors, as shown with in-house data | |
| Cost | | Disposable Cellbags add extra cost to production runs, but expense may be trade-off for less hands-on setup, cleaning labor. Also facility savings due to minimal utilities required |
| Scalability | | Single unit not available for >500 L |

large scale DNA purification, or the time-consuming work involved in stable line generation. BacMam viruses are baculoviruses engineered with mammalian expression cassettes, including a constitutive mammalian promoter [14]. They are generated just like any other baculovirus, and just like baculoviruses used for insect cell expression, have an excellent biosafety and environmental profile due to their inherent inability to replicate in mammalian cells. Furthermore, baculoviruses cannot be amplified in mammalian cells [15].

Gene delivery via BacMam virus transduction works well in a wide range of mammalian cells [14]. Many features have been

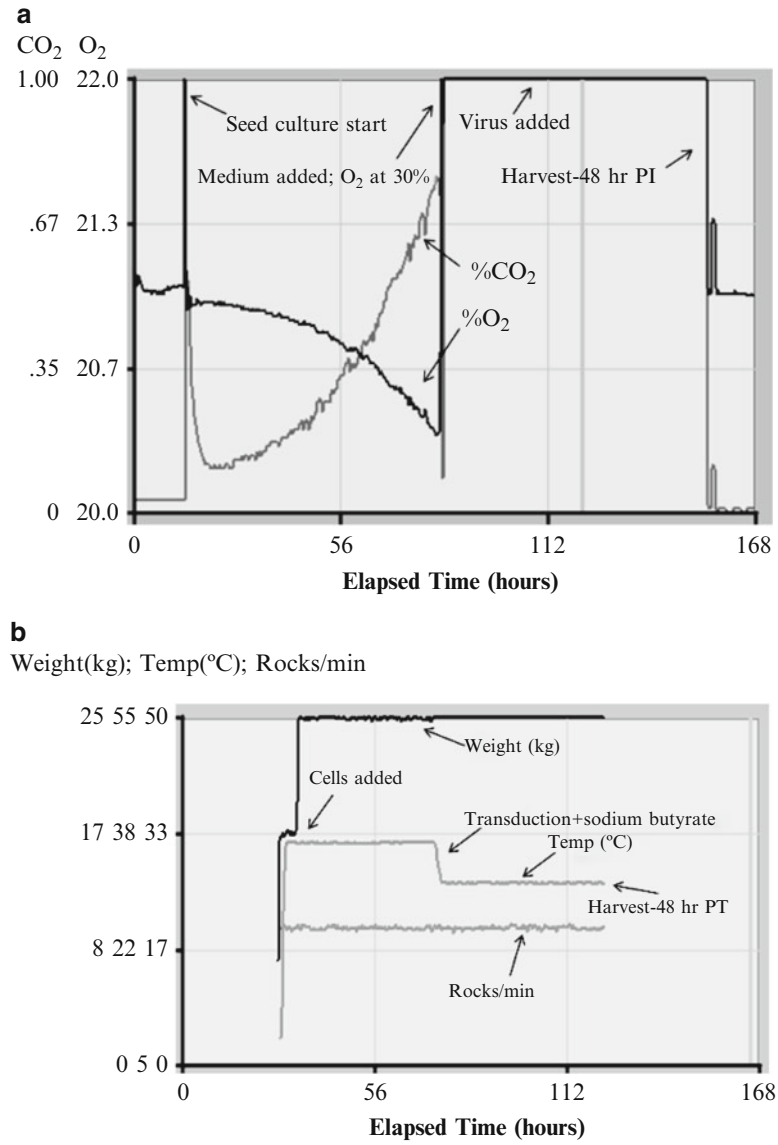


**Fig. 1** (**a**) The data from the mass spectrometer show oxygen consumption and carbon dioxide evolution immediately from the beginning of the run. Note the increase in oxygen concentration once the air mix is set at 30 % $O_2$. (**b**) SCADA data from CHO-GE production run. Note the shift in temperature from 37 to 31 °C at time of virus and sodium butyrate addition. This run was harvested ~48 h posttransduction

added to the vector design for BacMam viruses to improve expression and/or to increase the host range for transduction. We routinely use an engineered stable CHO suspension line with excellent growth features and high transduction efficiency for BacMam protein expression (*see* Subheading 2, **item 13**). As with all transient transfection protocols, many parameters can be adjusted to improve

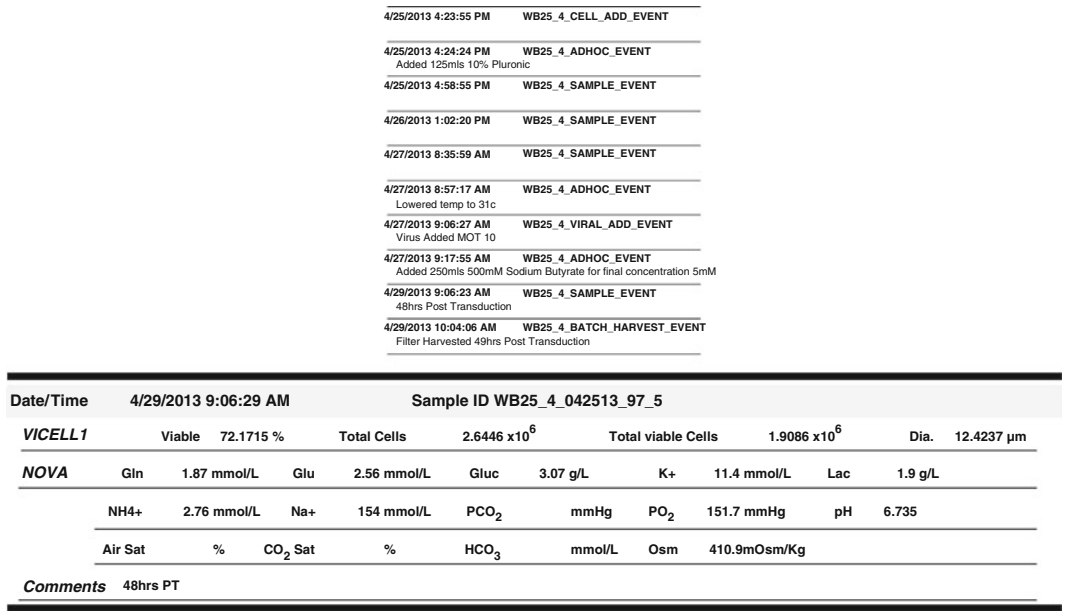


**Fig. 2** Time stamps document events throughout the run. Cell count, viability, and diameter data from the Vi-CELL and biochemical data from the Nova populate the batch record automatically

**Table 2**
**Parameters to evaluate to optimize recombinant protein expression**

| Parameter to test: | Default selection: |
|---|---|
| Cell line | CHO-GE |
| Cell density at transduction | 1–2 × $10^6$ cells/mL |
| Multiplicity of transduction | 20 |
| Sodium butyrate | 5 mM |
| Temperature shift | 31 °C |
| Time of harvest | 72 h posttransduction |

the yield. We have explored the use of histone deacetylase (HDAC) inhibitors to increase transgene expression from transduced BacMam viruses [16, 17], multiplicity of transduction (MOT) to increase copies of BacMam-delivered transgenes in cells [17], temperature shift during expression to induce growth arrest and prolong cell viability [18], and time of harvest. Selection of optimized conditions can be unique to the protein expressed, and it may be important to test each of these parameters in small scale (e.g., 150 mL shake flask studies). A summary of these parameters are listed in Table 2, with our default selections provided if time does not allow optimization of expression conditions.

**Table 3**
**Examples of signal sequences used for secreted protein expression**

| Native |
|---|
| CD33: Sialic acid-binding immunoglobulin (Ig)-like lectin (siglec3) |
| RAGE: human *r*eceptor for *a*dvanced *g*lycosylation *e*nd *p*roducts |
| BM40: Osteonectin |

BacMam expression in mammalian cells is particularly useful for secreted proteins [19]. Mammalian cell culture media should be serum-free to prevent purification problems, and as noted in Subheading 3.2, **step 1**, baculovirus stocks for secreted protein expression should be made without serum addition. The choice of signal sequence can be key to achieving high secreted protein expression levels, and although beyond the scope of this chapter, a list of signal sequences used by our laboratory is provided (*see* Table 3). Many of the proteins we express are used in animal studies and must contain as little endotoxin contamination as possible. The single-use, sterile filter harvest method described in Subheading 3.8.2 helps prevent introduction of endotoxin from glass or stainless steel equipment.

In this chapter, the use of baculoviruses for protein expression in insect cells and mammalian cells at the scale of 10–100 L is described using Wave Bioreactors. Cell inoculum preparation, baculovirus amplification, Wave Cellbag parameters, rock speed and angles, and harvest details are provided.

## 2 Materials

1. Wave Bioreactor System 20/50 (GE Healthcare Life Sciences, www.gelifesciences.com/) with Heater20/50 temperature control, LoadCell20 Weight Indicator, and a WAVEPOD II Integrated Controller.
2. The Wave Bioreactor System200 (GE Healthcare Life Sciences, www.gelifesciences.com/) with a Loadcell200 Weight Indicator and a DO200 dissolved oxygen and $CO_2$ monitor.
3. Cellbag bioreactors (GE Healthcare Life Sciences, www.gelifesciences.com/) available in many configurations or customized.
4. Sterile Tube Fuser (GE Healthcare Life Sciences, www.gelifesciences.com/) with interchangeable tubing holders that can be used for aseptic tube welding outside of a biological safety cabinet.
5. Hot Lips II brand tube sealer (GE Healthcare Life Sciences, www.gelifesciences.com/) that can be used for thermal sealing of fused/cut tubing.

6. Nova Bioprofile 400 (Nova Biomedical Corp., www.novabiomedical.com/) for monitoring the cell culture medium.
7. Vi-CELL XR® (Beckman Coulter, www.beckmancoulter.com/) for cell counting and determining cell viability via an automated trypan blue exclusion method.
8. CARR® ViaFuge® Pilot 9004 (Pneumatic Scale Angelus, www.psangelus.com/) for cell harvesting.
9. Disposable tubing set used for cell harvest (custom order from STI Components, Inc., www.stiflow.com/).
10. Mass Spectrometer, Prima dB (Thermo Scientific, www.thermoscientific.com/) for off gas analysis.
11. *Spodoptera frugiperda* (Sf-9) insect cells (American Type Culture Collection, Rockville, MD). The cells were adapted in-house to Hyclone SFX-Insect medium.
12. Hyclone SFX-Insect medium (Thermo Scientific, www.thermoscientific.com/). Medium purchased in custom bags made for welding to Cellbags.
13. Engineered stable CHO suspension line (CHO-GE). Though a number of cell lines, such as U-2 OS and HEK cells, have been used as hosts for BacMam-mediated gene expression [20]; in our experience CHO cells have yielded inconsistent transduction results. We decided to take a cell-engineering approach to this problem by identifying gene products that when expressed in CHO cells would lead to enhanced expression from BacMam-transduced expression cassettes. The Gam1 protein was first identified as a novel anti-apoptotic protein encoded by the CELO adenovirus [21]. Gam1 was shown to interfere with histone deacetylase activity, and furthermore its expression in cells results in increased gene expression from a number of promoters [22, 23]. Another multi-functional viral protein that has been shown to regulate host cell and viral transcription is the adenovirus E1A protein [24]. The expression of E1A in CHO cells has been shown to result in increased production of recombinant proteins [16, 25]. We developed a stable cell line derivative of CHO K1 cells that expresses these two protein modulators of gene expression by sequential introduction of a GAM1 expression cassette followed by an Ad5 E1A expression cassette. At each step clones were selected based on their ability to be efficiently transduced in suspension by BacMam-GFP virus. This has resulted in the CHO-GE (CHO-Gam-E1A), our GSK proprietary cell line that serves as our workhorse CHO cell for BacMam transduction (Condreay JP, Clay WC and Kost TA, unpublished data). Other robust suspension lines have also been developed and used for BacMam transduction. Examples are the CHO-S cell line from

Life Technologies (Life Technologies, www.lifetechnologies.com/) [26] and the HEK-293F cell line (Life Technologies, www.lifetechnologies.com/) [26].

14. MR1-4 CHO medium, a GSK proprietary formulation. Other commercial options for media are available, including CD CHO, Freestyle CHO Expression Medium for CHO lines and for HEK-293F cells, CD 293 or Freestyle HEK293 Expression Medium (all from Life Technologies, www.lifetechnologies.com/).
15. Sodium butyrate (Sigma-Aldrich, www.sigmaaldrich.com/) for BacMam transductions.
16. Climo ISF1-X Shaker (Kuhler, www.kuhner.com/), a stackable incubator shaker.
17. 2 L and 3 L (and other sizes) Erlenmeyer (Fernbach design) polycarbonate disposable flasks with vented caps (Corning, www.corning.com/) for growing cells.
18. Nalgene Rapid-Flow Filter units, with sterile 0.2 μm Polyethersulfone membranes (Nalgene, www.nalgene.com/) to clarify volumes of P2 baculovirus stocks below 2 L.
19. Polycap 36 SPF (Polyethersulfone membrane with glass microfiber prefilter) (Whatman, www.whatman.com/) in line with a Supor AcroPak 200 capsule filter (Pall, www.pall.com/) to clarify volumes of P2 baculovirus stocks above 2 L.
20. Sterile alcohol pads and 10 cc sterile syringes for sampling bioreactors.
21. Presterilized and pre-assembled bags in a range of sizes (300 mL, 1 L, 2 L and 5 L) with a central port for liquid addition and C-flex tubing for welding to Wave Cellbags (Fisher Scientific, www.fishersci.com/). A stainless steel stand for supporting the bags during liquid addition inside a biological safety cabinet is available (STI Components, Inc., www.stiflow.com/). These bags are used for cell and virus addition.
22. 0.5 μm Ultracap Protec Borosilicate Glass (GF) Filter Cartridge and 0.2 μm Ultracap STyLUX® PES disposable filter assembly (Meissner, www.meissner.com/) used to remove cells from supernatant containing secreted proteins. Supernatant is first pumped through the 0.5 μm filter and then further clarified by passing through the 0.2 μm filter. *See* **Note 1** about choice of filters.
23. Harvest bags (10, 20, 50, and 100 L with flange port) (STI Components, Inc., www.stiflow.com/) for collecting supernatants containing secreted proteins. *See* **Note 2**.
24. Hyclone Standard Drum SV50517.05 with bottom port, 100 L (other sizes are available) (Thermo Scientific, www.thermoscientific.com/) for supporting harvest bags during filling.

25. The GSK Fermentation Facility has implemented a SCADA system that provides operator interface with all the fermentation equipment for the purposes of monitoring, controlling, and batch reporting of fermentation runs. The SCADA system software is the Proficy HMI/SCADA-iFIX 5.5 product by GE Intelligent Platforms. (Our integrator was Marion White PE, Inc., Charlotte, NC, http://www.fixtraining.com/).
26. MatrikonOPC Server for Nova ASTM. (MatrikonOPC, www.matrikonopc.com). This allows data from the Nova bioanalyzer to be imported into the MS Access database.
27. Win911 (WIN911.com) provides remote alarm notification and acknowledgement capabilities.

## 3 Methods

### 3.1 Preparation of Cell Inoculum

#### 3.1.1 Insect Cells

1. Seed Erlenmeyer (Fernbach design) flask(s) with Sf-9 cells at $3 \times 10^5$ cells/mL and grow at 27–28 °C with shaking at 100 rpm. We use up to 750 mL and 2 L in 2 L and 3 L flasks, respectively, with excellent growth and viability (>95 %) and use Hyclone SFX-Insect medium.
2. Determine cell density and viability daily.
3. Passage the cells when their density reaches approximately $2–4 \times 10^6$ cells/mL.
4. Transfer cells to new flasks when there is a ring buildup at the liquid interface.

#### 3.1.2 Mammalian Cells

1. Seed Erlenmeyer (Fernbach design) flask(s) with CHO-GE cells at $3 \times 10^5$ cells/mL and grow at 37 °C, 5 % $CO_2$ and 80 % humidity with shaking at 100 rpm. We use up to 500 mL and 2 L in 2 L and 3 L flasks, respectively, with excellent growth and viability (>95 %) and use MR1-4 CHO medium.
2. Determine cell density and viability daily.
3. Passage the cells when their density reaches approximately $2–4 \times 10^6$ cells/mL.

### 3.2 Large-Scale Baculovirus Amplification: P2 Stocks

1. Prepare virus stocks by infecting Sf-9 cells at $10^6$ cells/mL with P1 virus at a multiplicity of infection (MOI) of 0.1. 5 % Heat-Inactivated Fetal Bovine Serum (FBS) is added at infection for long-term stock stability. However, if the desired expressed protein is secreted, then FBS should not be added since it would interfere with protein purification due to the contaminating serum proteins. Thus, for secreted protein production runs, virus stocks should be made fresh without FBS and should be used within a few weeks.

2. Monitor the infected cells with a Vi-CELL, which provides information about cell density, cell viability, and cell size. The cell size feature is particularly useful in monitoring infected cells since the virus infection causes cell size to increase; thus, increased cell size can be tracked as an indicator of infectivity [27].
3. Harvest cells when the cell viability drops to ~70 % (at ~72 h post-infection) by centrifuging at 4 °C at 900 × *g* for 30 min.
4. Filter the supernatant from **step 3** either through Nalgene Rapid-Flow Filter Units (PES) for low volume stocks (<2 L) or through a Polycap 36 SPF glass filter (prefilter, 5 μm) in line with a Supor AcroPak 200 capsule filter for higher volume stocks (>2 L). We have found that this approach removes lysed cell material without changing the virus titer. Clarified stocks are stored in sterile bottles at 4 °C in the dark. We have found that virus stocks prepared with 5 % FBS can be stored for up to 2 years, at which time we discard if not used.
5. Titer virus stocks. At GSK virus stocks are titered using the Baculotiter Kit developed by Invitrogen. Information on the method is available at www.invitrogen.com, but the kit is no longer sold by Invitrogen. Alternatively, any titer method can be used, e.g., the methods described in Chapters 4, 5, 10, 11, and 22 in this book. It is essential to titer all virus stocks. Average titers range from $5 \times 10^8$ to $10^9$ pfu/mL. For our processes, this titer is high enough to use stocks directly without concentration (*see* **Note 3**).

### 3.3 Setup of the Wave Cellbag, Insect Cells, and Mammalian Cells

Clean the rocking platform on the Wave units with 70 % Isopropanol. For the Wave System200, the inside of the lid is also wiped down. Note that the large red Emergency Stop button on the front panel should be used if the unit needs to be stopped rapidly.

The Cellbag name denotes the entire volumetric size of the bag, not the production volume. The maximum level the bags can be filled is one half the volumetric amount (e.g., a Cellbag50 can only be filled with medium to 25 L). Gas exchange headspace is provided by the remaining volumetric expansion.

The Wave System20/50 unit has two separate platforms that are easily interchangeable depending upon the size of Wave Cellbag that is used. One platform is used for Cellbag20s and a larger one is used for Cellbag50s. Each platform has corresponding heating pads, which must be used to maintain the temperature if reactors are not in a temperature-controlled cabinet or room.

There are at least two 0.2 μm filters provided on all standard Wave Cellbags. One is used as an air inlet filter and is attached to the air line, and one is used as an exhaust filter. The exhaust can be connected to a mass spectrometer for analysis of the off gas.

A heater jacket is snapped in place around the exhaust filter to decrease condensation build up. The third filter on the Cellbag100 and 200 is a spare filter to be used if the exhaust filter becomes clogged, and should be clamped off unless needed.

Controllers for Wave units can provide mixing of inlet air with facility provided $CO_2$ or $O_2$ to a set concentration that is directly supplied into the Cellbag.

### 3.3.1 Setup of Wave System200

1. Placement of the Cellbag: Turn cams inside the Wave lid counterclockwise to open Cellbag holders. Slide the edges of the Cellbag with the fiberglass rods into the holders and turn cams clockwise to lock. Smooth creases to the edges. Two Cellbag100s can be run at the same time in this unit, with the same temperature, rock angle, and speed.
2. Connect the ventilation air line from the air out port on the control panel to the left filter on the Cellbag. If using two Cellbag100s, then attach a separate airline to each.
3. Place the filter on the right side in a heater jacket. Place the heater plug into the heater jack located in the top of the lid. This will be the exhaust filter. Adjust defroster ducts in Wave lid to point down on the exhaust filter. If using two Cellbag100s, then place a heater jacket on the exhaust filters on each bag.
4. Open the inlet filter clamp.
5. Switch airflow on and set the airflow to 5 L/min to inflate the Cellbag. Once filled, the Cellbag should be tight, and creased minimally. Monitor the fill. The unit has a preset high pressure that will not allow over-inflation of the bag. Once this limit is reached, the monitor will flash "high pressure" and the airflow will stop until pressure falls below this high limit.
6. During the inflation step, move the harvest tube line inside the Cellbag (3/8″ID × 5/8″OD dip tube) toward the back left corner of the bag for future harvest.
7. Reduce airflow to 2 L/min once bag is filled.
8. A dissolved oxygen (DO) probe (if used) must be calibrated according to the 2 point-method outlined by GE Healthcare. Set the zero oxygen calibration and 100 % air saturation calibration with the temperature compensation at room temperature when calibrating to ambient air and with the zero oxygen calibration solution at room temperature. After the probe is threaded into the Oxywell2 sheath on the Cellbag, change the temperature compensation to the temperature of the culture and reset the 100 % air saturation. While handling the probe, do not touch the tip; rinse the probe with distilled water and shake it dry. Fill the Oxywell2 sheath with distilled water before threading in probe.

9. Set temperature, rock angle, and rock speed on the main screen. Turn on heaters (L and R) for a Cellbag200 or for two Cellbag100s or just one heater (L) for a single Cellbag100. Temperature control will begin with rocking.
10. Tare LoadCell.

#### *3.3.2 Setup of Wave System20/50*

1. Place Wave Cellbag on platform. Open cams by unscrewing on both sides of platform. Slide one rod in, tighten cams, slide opposite rod in, and tighten cams. Confirm that the bag is locked in place and free of creases.
2. Attach airline from the air pump out port to the inlet filter (filter without the restrictor) on the Cellbag. Snap filter heater jacket over the exhaust filter. Open both inlet filter clamps. Inflate the bag by switching on the air pump to 0.5 L/min. When filled, the bag should be tight without creases. Decrease the air flow to 0.2 L/min for a Cellbag20 or to 0.5 L/min for a Cellbag50. Test restrictor if air is not obviously flowing through.
3. If using a dissolved oxygen (DO) probe from Wave Biotech, then follow the instructions outlined in Subheading 3.3.1, **step 8**.
4. Set temperature, rock speed, and angle on platform with control panel.
5. Tare LoadCell.

### *3.4 Media Addition to the Bioreactor*

For each Wave Cellbag, one fifth of the final target volume is set as the starting seed volume. (Seed volumes: Cellbag20, 2 L; Cellbag50, 5 L; Cellbag100, 10 L, and Cellbag200, 20 L).

1. Prior to cell addition, use the Sterile Tube Fuser to weld the appropriate tubing on the inflated Cellbag to the tubing on media bag. Medium is added by gravity flow or a pump. On all Cellbag sizes, we use the 1/4″ID, 7/16″OD C-flex line for medium addition. When making additions to the Cellbag, the restrictor on the exhaust filter can be removed during the process to prevent pressure buildup. Monitor the temperature closely since the temperature may overshoot the set-point. The set-point can be increased stepwise to prevent temperature overshoot.
2. Use the LoadCell monitor on the Wave units to measure the medium to within 1–2 L of the desired volume (1 L = 1 kg).
3. Use the Hotlips Sealer to seal the media line when medium addition is complete. The tubing is then cut to detach the media bag. Take care to use as little of the C-flex line as necessary since other additions will be made through this line.

### 3.5 Cell Addition to the Bioreactor

#### 3.5.1 Sf-9 Cells for Insect Cell Production Run

1. For insect cells, ambient air alone (which can be supplied by the Wave unit or WAVEPOD II controller) without additional oxygen supplementation is sufficient with low density cell culture. If your process uses high density Sf-9 cell culture, then set the air mix to 30 % oxygen supplementation. Appropriate facility supply and Wave equipment are required for this supplementation.
2. Working in a biological safety cabinet, combine the appropriate amounts of Sf-9 cell inoculum (at ~$3 \times 10^6$ cells/mL) and media to a sterile addition bag to yield the total seed volume for the Cellbag at a cell density of $3 \times 10^5$ cells/mL.

#### 3.5.2 CHO-GE Cells for Mammalian Cell Production Run

1. For mammalian cells, 5–10 % $CO_2$ should be added to the air mix to control the pH of the medium. Appropriate house supply and Wave equipment are required for this supplementation. Check the pH of the medium after it is added to the bag, and then allow the medium to equilibrate with the $CO_2$/air mix in the headspace to the set temperature. This may take several hours, depending on the medium volume. Check the pH again before inoculation with cells.
2. Working in a biological safety cabinet, combine the appropriate amounts of CHO-GE cell inoculum (at ~$3 \times 10^6$ cells/mL) and medium to a sterile addition bag to yield the total seed volume for the Cellbag at a cell density of $3 \times 10^5$ cells/mL.

#### 3.5.3 Cell Addition and Monitoring, Both Cell Types

1. Weld the C-flex line from the bottom port of the addition bag onto the Cellbag for cell addition. This is also through the 1/4″ID, 7/16″OD C-flex line.
2. Use the Hotlips Sealer to seal the addition line after cells have been transferred to the Cellbag by gravity flow. Cut the bag free.
3. Table 4 lists recommended rock speeds, angles, and air flow. Monitor these settings closely. A clear surface "wave" action should be observed in the bag, but without intense force. Monitor for too much foaming and/or pockets of foam building up in corners; adjust rock speed/angle to prevent.
4. Monitor cell number (*see* Subheading 3.1). When the cell density reaches ~$3 \times 10^6$ cells/mL, add medium to obtain the final run volume by fusing tubing from the Cellbag to the media bag as described in Subheading 3.4. For Cellbag100 and 200 sizes, our medium is in custom large bags within barrels, with sufficient C-flex tubing to allow the use of pumps for medium addition.
5. Rock speed and angle settings are adjusted after medium addition. Suggested settings are shown in Table 5. Monitor run daily, and if the DO concentration drops below 50 % air saturation, then adjust the rock angle and then the rock speed, checking that an ideal wave is created without causing too much foaming.

**Table 4**
**Suggested settings for initial seed volumes**

| Cellbag | Seed volume | Cells/mL after seeding | Rock angle | Rock speed (rocks/min) | Airflow (L/min) | Temperature (°C) | $CO_2$ mix |
|---|---|---|---|---|---|---|---|
| *Sf-9 cells* | | | | | | | |
| Cellbag20 | 10 L | $0.6 \times 10^6$ | 8.5 | 18 | .5 | 27.5 | |
| Cellbag50 | 5 L | $0.3 \times 10^6$ | 8.5 | 28 | .5 | 27.5 | |
| Cellbag100 | 10 L | $0.3 \times 10^6$ | 7.5 | 15 | 2 | 27.5 | |
| Cellbag200 | 20 L | $0.3 \times 10^6$ | 7.5 | 15 | 2 | 27.5 | |
| *CHO-GE cells* | | | | | | | |
| Cellbag20 | 10 L | $0.6 \times 10^6$ | 8.5 | 18 | .5 | 37 | 5 % |
| Cellbag50 | 5 L | $0.3 \times 10^6$ | 10 | 20 | .5 | 37 | 5 % |
| Cellbag100 | 10 L | $0.3 \times 10^6$ | 7.5 | 15 | 2 | 37 | 5 % |
| Cellbag200 | 20 L | $0.3 \times 10^6$ | 7.5 | 15 | 2 | 37 | 5 % |

**Table 5**
**Suggested settings for final production volumes**

| Cellbag | Final media addition | Rock angle | Rock speed (rocks/min) | Airflow (L/min) | Temperature (°C) | $CO_2$ mix |
|---|---|---|---|---|---|---|
| *Sf-9 cells* | | | | | | |
| Cellbag20 | | 8.5 | 18 | 0.5 | 27.5 | |
| Cellbag50 | 20 L | 8.5 | 28 | 0.5 | 27.5 | |
| Cellbag100 | 40 L | 8.5 | 24 | 2 | 27.5 | |
| Cellbag200 | 80 L | 8.5 | 24 | 2 | 27.5 | |
| *CHO-GE cells* | | | | | | |
| Cellbag20 | | 8.5 | 18 | 0.5 | 37 | 5 % |
| Cellbag50 | 20 L | 10 | 20 | 0.5 | 37 | 5 % |
| Cellbag100 | 40 L | 8.5 | 20 | 2 | 37 | 5 % |
| Cellbag200 | 80 L | 8.5 | 20 | 2 | 37 | 5 % |

### *3.6 Virus Addition*

#### *3.6.1 Infection: Insect Cell Protein Production*

1. Add virus to Sf-9 cells when cells reach approximately $1–2 \times 10^6$ cells/mL. The cells should have a viability greater than 95 %. In a biological safety cabinet, add the appropriate amount of virus stock at an MOI of 1 to a sterile addition bag with a C-flex line attached to the bottom port. The 1/4″ID, 7/16″OD C-flex line on the Cellbags is also used for virus addition.

*3.6.2 Transduction: Mammalian Cell Protein Production*

1. For CHO-GE cells, virus is added as described above at an MOT of 20 when cells reach approximately $1–2 \times 10^6$ cells/mL, with viabilities greater than 95 %. *See* Table 2 for parameters to test. Also listed are our default selections, including adding sodium butyrate to 5 mM and lowering the temperature to 31 °C with the addition of virus.

*3.6.3 Virus Addition to Cellbag, Both Cell Types*

1. Use the Sterile Tube Fuser to fuse the C-flex tubing by connecting the virus addition bag to the Cellbag. Virus is added by gravity flow or by pumping into Cellbag.
2. After virus addition, seal the C-Flex line with the Hot Lips sealer and cut in the middle of the seal to remove the addition bag.

### *3.7 Monitoring During Production Run*

On all Wave Cellbags, a CLAVE© sampling connector is provided for aseptic sampling outside a biological safety cabinet. This connector has a luer lock fitting to accommodate a needleless sterile syringe for sampling.

1. Stop the rocking prior to removing a sample from the Cellbag. If using the Wave 200 unit, then there is a "Sample" setting on the control monitor that will change the angle of the platform to tilt forward to aid in drawing a sample through the sampling connector.
2. Remove the cap on the sampling connector, and wipe the port with a sterile alcohol pad. Attach a needleless syringe (10 cc) onto the connector. While slightly pulling out on the syringe plunger, open the clamp on the tubing of the sampling connector, hold the blue area of the sampling connector, and draw 10 mL into the syringe. Close the clamp. Remove the syringe. This sample will be discarded, as it was pulled to clear cells from the sampling line.
3. Wipe the port with a new sterile alcohol pad. Attach a new sterile needleless syringe onto the connector. Turn the rocking on for a few min, and then stop the rocking. Repeat the sampling steps above using a new syringe. After wiping the port clean, replace the cap to the luer fitting on the sampling connector and restart the rocking.
4. Analyze the sample, e.g., with a Nova Bioprofile 400. Essential parameters to monitor include pH, $pO_2$, and % air saturation for insect cells, and pH, $pO_2$, $pCO_2$, and % air saturation for mammalian cells. Other parameters that can be monitored if desired include glucose, glutamine, glutamate, and lactate. For valid readings on the Nova instrument, samples must be analyzed as quickly as possible. Alternatively, a Nova automatic sampler can be used.

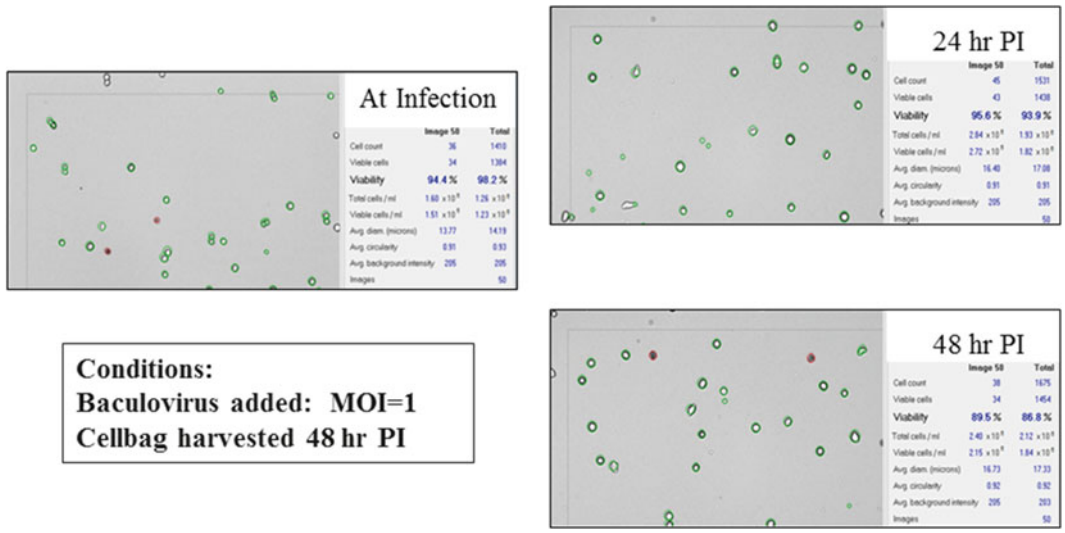


**Fig. 3** ViCell images of Sf-9 cell production run. The live cells are the *circled*, *open cells* and the dead cells are the *circled solid*, *dark cells*. On the instrument, these are shown as *green* (live) and *red* (dead) *circles*

5. It is also possible to have off-gas analysis performed using a mass spectrometer. E.g., a Prima dB instrument can be used to monitor for $O_2$ consumption and $CO_2$ evolution. Data can be used to monitor growth rate and to compare with other runs. Also, a steep change in the slope may suggest microbial contamination.
6. Place approximately 2 mL of sample on 60 mm disposable Petri dish to visually inspect the culture under a microscope.
7. For cell counts using the Vi-CELL XR, 0.5 mL of culture is required in a provided sample cup. Select the appropriate cell type and start the analysis. Typically, 50 images are analyzed for each sample. Images and/or Excel data can be exported. Examples of Vi-CELL data generated during Sf-9 and CHO-GE protein production runs are shown in Figs. 3 and 4, respectively.

### 3.8 Harvesting

Our standard protocol for Sf-9 protein production runs is a harvest time of 48 h post-infection, and a harvest time of 72 h posttransduction for CHO-GE runs. Occasionally, based on the result of small scale expression testing with samples collected at 24, 48, and 72 h to validate new P1 stocks, a different ideal harvest time is identified. This was the case in the CHO-GE run shown in Figs. 1b and 2.

#### 3.8.1 Cell Harvest, Insect or Mammalian, for General Protein Purification Use

1. Cells are harvested using a continuous flow centrifuge by following the manufacturer's installation specifications and procedures. To begin the harvest, rocking is stopped on the Wave unit. If using DO probes from Wave, then remove from the Cellbag and store properly. Disconnect the airflow line and clamp all filters.

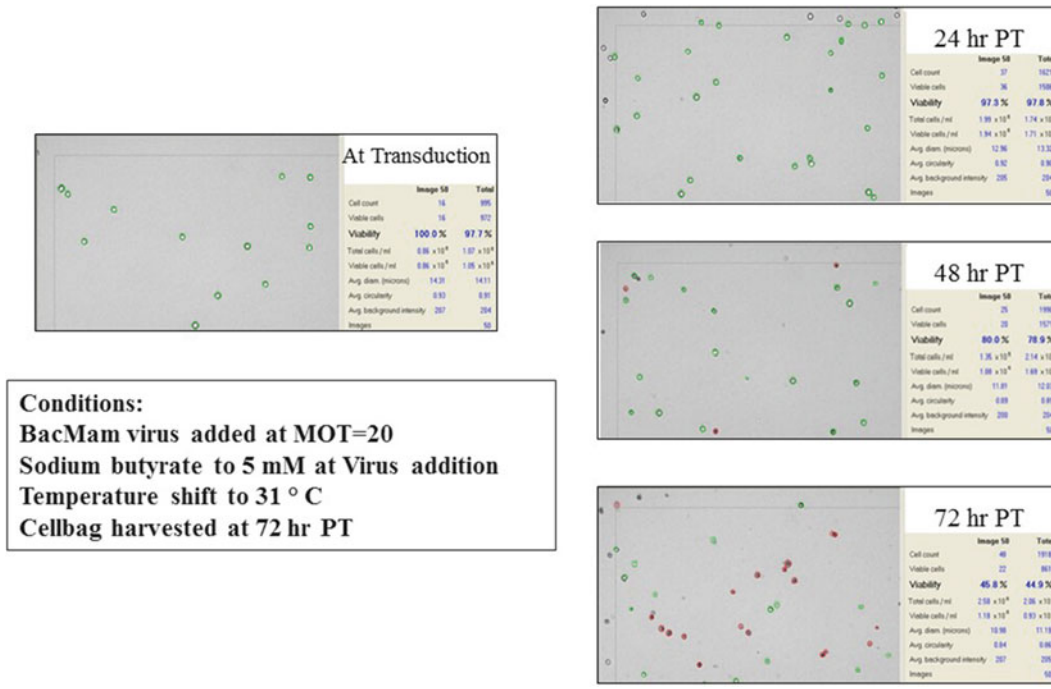


**Fig. 4** ViCell images of CHO-GE production run. The live cells are the *circled*, *open cells* and the dead cells are the *circled solid*, *dark cells*. On the instrument, these are shown as *green* (live) and *red* (dead) *circles*

2. The Wave200 has a "HARVEST" position selection on the display panel, and this will tilt the platform back to aid flow from the Cellbag. This harvest line is located in the back left corner on the Cellbag100s and 200s. The Cellbags will collapse completely as they empty.
3. A disposable tubing set used for cell harvest was made to our specifications to quick-connect to the harvest line on the Cellbag100s and 200s. For the Cellbag50s, an adapter section of 1/4″ID, 7/16″OD C-flex with a quick-connect is welded onto the Cellbag line to snap into the harvest rigging set.
4. The disposable tubing set used for cell harvest provides a length (7 ft) of silicon tubing (1/2″ID × ¾″OD) for use with a peristaltic pump to move the cell culture from the Cellbag into the pre-chilled Viafuge. This is connected to the Viafuge feed hose via a ½″ Colder barb-lock mini-sanitary fitting and a tri-clover clamp.
5. The harvest rigging set also provides 2 × 3′ lengths of 3/8″ID × 5/8″OD C-flex tubing with quick connect caps. One is used as the buffer hose to feed buffer from a carboy into the Viafuge. We use 1× PBS. The other is used as a cell harvest line to pump cell concentrate from the Viafuge bowl into a chilled collection bottle. Each hose has a clamp to close off as necessary during the harvest procedure.

6. If the production run is for a secreted protein and the possibility of endotoxin contamination is not an issue, then the medium can be collected through the Viafuge centrate outlet (*see* **Note 4**).
7. If the production run is for an intracellular protein, then the Viafuge centrate outlet is connected via tubing to a decontamination tank.
8. The Viafuge process setting used is 2000 × *g*, with an input pump flow rate of 1.8 L/min. To ensure that all cells are retained, it is important to determine the process setting and the pump flow rate specific to the pump and tubing used, as well as the cell type and density. During high-cell density harvests, it is important to check the centrate periodically for cell loss.
9. To remove cell concentrate from the Viafuge bowl, the pump speed is stopped and reversed to transfer cells into a harvest bottle held on ice. The volume of the Viafuge bowl is 1.8 L. Care is used to keep harvested cells chilled to prevent protease activity.
10. When the cell concentrate has been pumped into the bottle, the Viafuge bowl is washed with 1× PBS and the contents of the bowl are collected again.
11. Cells from the collection bottles are poured into 1 L Nalgene centrifuge bottles and centrifuged at 900 × *g* in a Sorvall RC-3C Plus at 4 °C for 30 min. The supernatant is poured from the cells and the pellets combined into one bottle and washed with chilled PBS. If desired, protease inhibitors can be added to the PBS buffer. The pellet is centrifuged at 900 × *g* as above, the wash buffer is poured off the pellet, and using as little PBS as is necessary, the pellet is moved to a storage bag, flattening the bag and releasing air. Pellets are weighed and frozen at –80 °C (*see* **Note 5**).

*3.8.2 Secreted Protein (Supernatant) Harvest, Insect or Mammalian Protein Production Runs: Low Endotoxin Conditions*

To prevent introduction of endotoxin from the harvest equipment, only disposable, single-use, presterilized supplies should be used.

1. Prior to harvest, assemble a filter train using C-flex tubing matched to the harvest line size and type from the Cellbag. A length of tubing to bridge the Cellbag harvest line through an appropriate size pump is attached securely to a filter assembly using tie-wraps. This length of tubing will be welded to the Cellbag harvest line. In the GSK facility, Meissner Ultracap Single-Use Capsule Filter Assembly units are connected so that the supernatant from the Cellbag is first pumped through a prefilter, a 0.5 μm Ultracap Protec Borosilicate Glass (GF) Filter Cartridge, then through a sterilizing 0.2 μm Ultracap STyLUX® PES disposable filter assembly for volumes up to

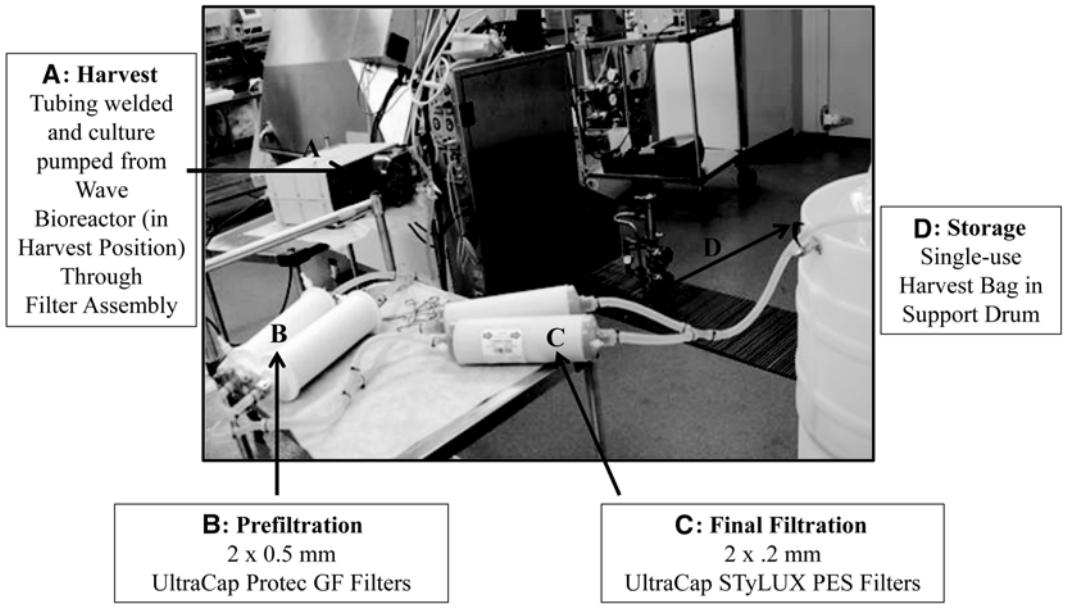


**Fig. 5** Low endotoxin harvest method: culture pumped from Cellbag, through filters set up in parallel, into sterile harvest bag

25 L. For harvests greater than 25 L, two of each filter type are connected in parallel to prevent filter fouling. To the end of the final sterilizing filter, C-flex tubing is attached to allow connection to the disposable storage bag at the time of harvest. The entire filter assembly should be autoclaved according to the manufacturers' guidelines. *See* Fig. 5 for a Cellbag200 harvest example.

### *3.9 Product QC*

The standard methods for analyzing protein production quality and quantity include Coomassie stained SDS-PAGE gels and Western blots. As in many laboratories, most of the recombinant proteins expressed in our facility include tags for purification purposes. Such tags can be used for small-scale analysis, using 1–2 g of harvested cell pellet. The cell pellet is lysed by brief sonication and purified using the applicable resin for a particular tag. Samples are saved throughout the procedure and run on gels along with the eluted purified protein sample. One set of samples is used for stained gels, and one set for Western blot analysis. Along with confirmation of expression, this also provides excellent information about the solubility of the recombinant protein. Time-course samples can also be collected and analyzed during the production run.

If the recombinant protein is secreted, then a 10–50 mL sample can be pulled via the sample port on the Wave Cellbag at harvest. Cells are pelleted by centrifugation, and the cell pellet washed with PBS. An aliquot of the conditioned medium can be used for loading onto gels. Protein can be purified using an applicable resin

incubated with the supernatant for 1 h if the protein is tagged. Following incubation, the resin is collected after centrifuging the supernatant. For a thorough analysis, the cell pellet can be lysed and also checked for expression.

If the conditioned medium is to be concentrated (*see* **Note 6**), then samples should be collected during this process to ensure that the expressed protein is not lost during the concentration.

The molecular weight of the purified protein can be determined by mass spectrometry. Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) is available to our group, to further confirm the protein identity.

In many cases, an activity assay can be used to confirm expression. Small-scale samples can be tested from purification preps or possibly even tested at the lysed cells or conditioned medium stage without purification.

## 4 Notes

1. Many other filter choices are available, which may be more suitable for specific cell processes and protein types. We use in-line 10-in. units with hose barb connections, a sanitary vent and ¼″ sanitary drain plug, and increase surface area by adding additional filters in parallel. If high density cell culture is used with cell densities over $5 \times 10^6$ cells/mL, then 5 μM depth filters (e.g., MicroCap Single-Use Capsules with cellulose and high purity diatomaceous earth filter media (www.ertelalsop.com/) should be used instead of 0.5 μM filters to prevent fouling). We tend to over-size the available surface of a filter train because having filters plug mid-harvest can compromise the sterility and/or endotoxin burden in the harvest. We have found on rare occasions that the membrane materials listed may bind the recombinant protein of interest. In these instances, an optimization of filter materials is performed on small-scale expression samples to ensure that the proper membrane material is used for the production harvest.
2. Having a bottom feed port on the harvest bag allows the contents to flow and empty without introducing air bubbles into protein purification columns.
3. With high baculovirus titers in the range we routinely obtain, virus addition volume is less than 10 % of the final culture volume. We have had no issues with viability in CHO-GE protein production runs with this amount of BacMam stock (in insect medium) added to the mammalian cell culture medium.
4. The prefilters listed in Subheading 2, **item 22** (also, *see* **Note 1**) can be used to clarify the supernatant harvested using a Viafuge before loading onto purification columns.

5. By freezing pellets in this manner, all the material does not need to be thawed at once. Portions can be broken off, weighed, and protein purification methods can be developed without waste.
6. If the harvested supernatant is to be concentrated using a tangential flow device such as a Pellicon Tangential Flow Filtration Cassette, then it is important to choose the correct membrane and molecular weight cut-off for your protein (www.millipore.com/) to prevent inadvertently losing recombinant protein. As described, it is important to collect and analyze samples from the process, including starting material, concentrate, and permeate, prior to discarding the permeate.

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# Chapter 13

# Protein Production with Recombinant Baculoviruses in Lepidopteran Larvae

**Elena Kovaleva and David C. Davis**

## Abstract

With an increasing need for functional analysis of proteins, there is a growing demand for fast and cost-effective production of biologically active eukaryotic proteins. The baculovirus expression vector system (BEVS) is widely used, and in the vast majority of cases cultured insect cells have been the host of choice. A low cost alternative to bioreactor-based protein production exists in the use of live insect larvae as "mini bioreactors." In this chapter we focus on *Trichoplusia ni* as the host insect for recombinant protein production, and explore three different methods of virus administration to the larvae. The first method is labor-intensive, as extracellular virus is injected into each larva, whereas the second lends itself to infection of large numbers of larvae via oral inoculation. While these first two methods require cultured insect cells for the generation of recombinant virus, the third relies on transfection of larvae with recombinant viral DNA and does not require cultured insect cells as an intermediate stage. We suggest that small- to mid-scale recombinant protein production (mg-g level) can be achieved in *T. ni* larvae with relative ease.

**Key words** *Trichoplusia ni*, Cabbage looper, Baculovirus, Recombinant protein expression, Transfection of insect larvae

## 1 Introduction

There are many published examples of the use of lepidopteran larvae for baculovirus-mediated protein production. The use of larvae was pioneered with the silkworm, *Bombyx mori* (e.g., α-interferon [1] and mouse interleukin-3 [2]), and this host insect continues to be extensively used both for research and for commercial protein production, e.g., by the Japanese firm Katakura (e.g., PON1 [3], protein complex [4] and 45 recombinant proteins from six categories [5]). Other lepidopteran hosts have also been investigated, including the tobacco hornworm *Manduca sexta* [6], the Cecropia moth *Hyalophora cecropia* [7], the beet armyworm *Spodoptera exigua* [8], the tobacco budworm *Heliothis virescens* [9], the saltmarsh caterpillar *Estigmene acrea* [10], and, probably most importantly, the cabbage looper *Trichoplusia ni*.

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*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is by far the most widely used baculovirus expression vector. While AcMNPV infects a wide range of lepidopteran hosts, not all moths are good hosts for this virus. For example, while being susceptible to a mutant [11], *B. mori* is not susceptible to wild type AcMNPV, and *M. sexta*, *H. cecropia*, and *S. exigua* are all much less susceptible than *H. virescens* [12]. *T. ni* is an excellent host for AcMNPV [13] and has been extensively used to produce a variety of proteins (e.g., insulin receptor kinase domain [14], adenosine deaminase [15], Phospholipase A2 [16], interleukin-2 [17], the cardiac sodium-calcium exchanger [18], Fab antibody [19], and phosphatase 2A subunit [20]).

While commercial-scale protein production in *T. ni* larvae is available (Chesapeake PERL Inc., Savage, MD, and Entopath, Easton, PA), this chapter is aimed at small to medium scale production on the bench-top. Compared to production in cultured insect cells, larvae can produce a large amount of protein at reduced cost and without the need for investment in expensive equipment [5, 21]. In addition, the scale-up issues typical of bioreactor-based production do not occur when using insect larvae [13]. Potential applications for proteins produced in larvae include use in vaccine preparation [22–24] and crystallography [25].

We limit the discussion in this chapter to production of protein in cabbage looper larvae, using three different methods that differ in the manner of larval inoculation. Traditionally, larva-mediated protein expression has been done by generating a recombinant AcMNPV vector in cultured cells (commonly, *S. frugiperda* cells), followed by budded virus amplification and the establishment of an infection by injecting the budded virus into late-instar larvae. This first method is effective but tedious: even a skilled investigator can inject only a few hundred larvae. A second method, useful for producing larger amounts of recombinant protein, is based on oral inoculation rather than injection. It is possible to generate orally infective inoculum, so-called pre-occluded virus (POV), by processing a few dozen injected larvae and to use this inoculum to infect thousands of *T. ni* larvae via the diet. Finally, in a third method the intermediate steps in cultured cells are eliminated completely by generating recombinant AcMNPV DNA (Bacmid) in bacteria and using this Bacmid DNA to transfect *T. ni* larvae (Liu and van Beek, unpublished results).

## 2 Materials

### 2.1 Rearing of Trichoplusia ni Larvae

1. We recommend the purchase of eggs or larvae from any of several commercial suppliers (Benzon Research, Carlisle, PA, www.benzonresearch.com; Bio-Serv, Frenchtown, NJ,

www.bio-serv.com; Entopath, Easton, PA, www.entopath.com) (*see* **Notes 1** and **2**).

2. Corn cob grits (e.g., Bio-Serv). A bulking agent that is needed only if the larvae are reared starting with "loose" *T. ni* eggs. If not sterilized, then autoclave before use.
3. General Purpose Lepidoptera Diet (#F9772, Bio-Serv, Frenchtown, NJ), a dry mix insect diet (*see* **Note 3**). We recommend purchase of insect eggs and diet from the same vendor.
4. Transfer forceps (e.g., BioQuip, Rancho Dominguez, CA: #4750): soft tweezers for handling of insect larvae.
5. Eight-oz cups and fitting lids (e.g., Solo Cup Company, Urbana, IL) for rearing larvae in groups of approximately 25 individuals (*see* **Note 4**).
6. Dissecting microscope with a micrometer scale engraved in one ocular lens (*see* **Note 5**).

### 2.2 Inoculation by Injection

1. *Spodoptera frugiperda* Sf-9 (ATCC: #CRL-1711) or Sf-21 cells can be used for generating recombinant virus, as well as for titration of the extracellular virus stock (*see* **Note 6**).
2. Insect cell culture media. There are many types of insect media commercially available, and any medium recommended by the manufacturer for maintaining Sf-9 or Sf-21 cells is suitable (*see* Chapter 8 and **Note 7**).
3. Ten-microliter syringe (Hamilton, Reno, NV), fitted with a 26 s gauge needle.

### 2.3 Oral Inoculation

1. Mortar and pestle.
2. FD&C Blue #1 (Hilton-Davis, Cincinnati, OH) or blue food coloring dye.
3. Plastic screen (100 mesh) or cheesecloth.

### 2.4 Transfection of Larvae with Bacmid DNA

1. Cellfectin II reagent (Life Technologies, CA): a transfection reagent.
2. Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI).

### 2.5 Homogenization and Clarification

1. Anti-melanizing agent: Either β-mercaptoethanol (BME) (Sigma-Aldrich, St. Louis, MO), or a stock solution of 25 mM phenylthiourea in ethanol (PTU) (Sigma-Aldrich) (*see* **Note 8**).
2. Complete Protease Inhibitor Cocktail tablets (Roche Applied Sciences, Indianapolis, IN).
3. Extraction buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA.

## 3 Methods

### 3.1 The Host Insect

The cabbage looper is a suitable and widely used insect for recombinant protein production. When the insects are reared on a suitable diet at their optimal temperature (~29 °C), either in the dark or under a day/night regimen, they develop from egg to pupa in approximately 13 days. After about 2 days in the egg stage, larvae will hatch and progress through five instars over a period of about 9 days. Late in the fifth instar, larvae change in appearance when they enter the prepupal stage, which lasts approximately 2 days. Metamorphosis takes place in the pupal stage, and adult insects emerges after about 4 days.

### 3.2 Diet Preparation

Prepare the diet as recommended by the supplier. As an example, the following is a slightly modified version of the manufacturer's protocol for preparation of General Purpose Lepidoptera Diet.

1. For 1 L diet: add 17 g agar to 400 mL water and mix thoroughly.
2. Heat until boiling, e.g., in a microwave. Stir the mixture occasionally during the process. The suspension should appear white and foaming when boiling.
3. Add 160 mL cold water and mix.
4. While the agar is being heated, mix the bulk diet ingredients (144 g) in 300 mL cold water.
5. Combine the agar and the bulk diet ingredient suspensions, top off with water to 1 L, and mix thoroughly.
6. Before the diet sets at approximately 37 °C, pour ~20 mL diet into each 8-oz cup and let stand for 15 min to allow the diet to solidify and the condensate to evaporate.
7. Close the cups with the proper lid. Freshly prepared diet may be stored under refrigeration for 3 weeks without any detrimental effects. Remember to equilibrate the diet to room temperature for at least 1 h and let condensed water evaporate before placing insects or eggs on the diet.

### 3.3 Trichoplusia ni Rearing

*T. ni* can be purchased as surface-sterilized eggs attached to a substrate such as paper towel or muslin (Subheading 3.3.1), as "loose" eggs (Subheading 3.3.2), or as larvae on diet (Subheading 3.4).

#### 3.3.1 Larval Rearing Starting with Eggs on a Substrate

1. Cut the substrate into small strips (50–100 eggs). Egg density can be estimated by counting under a dissecting microscope. To this end mark an area of approximately 1 $in^2$ and count under the lowest magnification.
2. Staple a strip to the inside of the lid of each 8-oz plastic cup filled with 20 mL diet.

3. Incubate at 29 °C.
4. After 3–4 days of incubation, begin to monitor larval development as described under Subheading 3.4.

#### 3.3.2 Larval Rearing Starting with Loose Eggs

1. Weigh out the corn cob grits to be used (1 g grits per 8-oz cup).
2. Add 1 mL water for each 15 g grits and mix until the clumps have disappeared.
3. Mix insect eggs with the moistened corn cob grits. Needed per cup: 1 g corn cob grits and 10–15 mg eggs (1 mg contains ~10 eggs).
4. Incubate the mixture for 16–24 h at 29 °C.
5. For each cup, spread approximately 1 g egg-grits mixture onto the diet. The diet should be at room temperature and show no excessive moisture on its surface.
6. Incubate at 29 °C.
7. After 3–4 days of incubation, begin to monitor larval development as described under Subheading 3.4.

### 3.4 Determination of Developmental Stage

*T. ni* larval development consists of five instars, which are developmental stages separated by a molt. Since injection of larvae is carried out preferentially during early fifth instar, and oral inoculation at late fourth instar, it is important to be able to determine the developmental stage of the larvae; this is also convenient for planning experimentation. It is possible to manipulate developmental speed by changing incubation temperature.

1. Determine the instar of the larvae at 3 or 4 days after seeding the eggs onto the diet. To this end, take a small sample of larvae and measure their head capsule width under a dissecting microscope with a micrometer scale engraved in the ocular (find the correct magnification via calibration with the aid of a ruler). Determine the larval instar after comparison of measured and tabulated values (Table 1). Larval weight is also an indicator of developmental stage, albeit a less reliable one. When using weight it is important to note that larvae early in a particular stage are lighter than they were late in the preceding stage (Table 1).

**Table 1**
**Weight and head capsule width of *Trichoplusia ni* larvae**

| Insect stage (instar) | First | Second | Third | Fourth | Fifth |
|---|---|---|---|---|---|
| Head capsule width (μm) | 292 | 440 | 700 | 1230 | 1900 |
| Weight (mg) | 0.1–1.5 | 1.4–11.0 | 10.2–16.5 | 15.4–74.0 | 70.0–350 |

2. Estimate the number of larvae per cup and reduce to approximately 25 (*see* **Note 9**).
3. At 29 °C it will take 6–7 days between seeding of the eggs and the molt from fourth to fifth instar (*see* **Note 10**). Daily monitoring of the larvae will help in choosing the correct stage for inoculation. Whether the larva is early or late in a particular instar can be judged by the width of the head capsule in relation to the width of the body. Larvae that have recently molted possess a relatively wide head capsule and slender body, whereas late in the instar the width of the body exceeds that of the head.

### 3.5 Recombinant Virus

The construction of recombinant baculovirus vectors is described elsewhere in this book (Chapter 4), and a number of different baculovirus vector cloning kits are available from various commercial sources (e.g., Invitrogen, BD Biosciences/Pharmingen, Novagen, NextGen Sciences, and AB Vector). The kits introduce heterologous coding sequences either by homologous recombination or transposition insertion. It is possible to eliminate bacterial amplification of a transfer vector plasmid and/or bacmid and to introduce heterologous coding sequences by direct ligation into a baculoviruses modified with unique restriction endonuclease cut sites (e.g., *see* [26]). This direct ligation method has been successfully used to generate customized baculovirus vectors for the whole insect platform (Chesapeake PERL Inc., Savage, MD). The choice of baculovirus cloning vector depends on availability, ease of the procedures (screening), flexibility (number and types of expressible promoters, availability of purification tags and secretion signals, etc.), or even on the presence/absence in the vector of a virally encoded protease or chaperones. However, the choice of a particular system is not influenced by whether the recombinant virus will be used in cultured cells or in larvae; thus, each system will yield recombinant AcMNPV suitable for infection of *T. ni* larvae and the expression of recombinant protein. *See* **Note 11** for safety aspects of recombinant baculoviruses.

### 3.6 Inoculation

There are three different methods for inoculating larvae: (a) injection with extracellular virus; (b) oral inoculation; and (c) transfection with viral DNA. Injection with extracellular virus is used most commonly. If a large amount of recombinant protein is desired, or if the yield of the target protein is expected to be relatively low (as may be the case with membrane proteins), then large numbers of larvae may be needed to produce the desired amount of protein. Injection of thousands of larvae can be avoided by using a small number (e.g., 20) of injected larvae to prepare orally infective inoculum for a mass inoculation. The orally infectious virus morphotype in preoccluded virus (POV) inoculum consists of virions that are produced late in the infection cycle and remain in the

nucleus as they are destined to be incorporated into polyhedral occlusion bodies [27–29]. However, almost all recombinant baculovirus expression vectors lack the polyhedrin gene and therefore no polyhedral occlusion bodies are formed.

1. *Extracellular virus.* Inoculum consisting of extracellular virus (also referred to as budded virus) is obtained by collecting the medium from infected cell culture. The working stock of recombinant AcMNPV for injection of larvae consists of extracellular virus with a titer of $10^6$ pfu/mL or higher. Methods for extracellular virus titration are described elsewhere in this volume (Chapters 4, 5, 10, 11 and 22).
2. *Preoccluded virus.* The working stock of recombinant AcMNPV for oral inoculation consists of POV. This form of the virus cannot be titered in vitro. Its potency can be determined only by larval bioassay, but this is usually not necessary. POV inoculum is most easily prepared from a small batch of larvae that have been injected with extracellular virus.
3. *Recombinant viral DNA.* The third inoculation method, larval transfection, is accomplished with viral DNA, in the form of *Escherichia coli*-produced Bacmid (Bac-to-Bac™, Invitrogen, Carlsbad, CA) (for an alternative method *see* **Note 12**). DNA is purified from the bacterial culture using a miniprep method, and DNA concentration in the inoculum can be estimated, e.g., by spectroscopy.

### 3.6.1 Larval Injection

Larval injection may seem difficult at first, but after a little practice (and the appropriate syringe with a sharp needle) it appears to be remarkably easy on the insect. Early fifth instar larvae are injected with approximately 1000 pfu of extracellular virus. This dose leads to a synchronous infection in all treated larvae. Typically, a small droplet of hemolymph will appear over the wound site immediately after injection. The hemolymph will melanize over a period of a few hours and wound healing takes place underneath; however, some larvae (usually less than 10 %) do not survive inoculation. Control larvae will pupate after 2–3 days, but in virus-infected larvae pupation (in fact, any molt) is blocked by a virus-encoded ecdysteroid UDP-glucosyltransferase [30]. Infected larvae will die from the virus infection after approximately 3–3.5 days (or, if lower doses are used, after as long as 4–5 days).

1. Prepare control inoculum (insect medium), and viral inoculum consisting of extracellular virus at a titer of $10^6$ pfu/mL (if necessary dilute stock with insect medium).
2. Select 100 early fifth instar larvae (head capsule width 1.9 mm, weight about 110 mg). Leave the selected larvae on their diet, until ~10 min before inoculation, when they may be cooled on ice in groups of 5 larvae (*see* **Note 13**).

3. Inject four groups of five larvae with 1 μL control inoculum as follows: wearing latex gloves, hold the insect lightly between thumb and forefinger and insert the needle at a low angle (to avoid puncturing the insect's midgut) into the body cavity at the posterior half of the larva. Release the insect, inject about 1 μL inoculum by advancing the plunger, and then slide the larva carefully off the needle onto fresh diet.
4. Repeat **step 3** for the remaining 80 larvae using the recombinant budded virus stock.
5. Incubate the larvae at 29 °C.
6. After approximately 24 h, remove any larvae that died from the injection procedure.
7. Harvest infected larvae when approximately 10 % of the remaining insects are dead, typically between 72 and 84 h after inoculation. If the larvae are not to be processed immediately, then they should be stored at −80 °C.

### 3.6.2 Oral Inoculation

1. Follow the procedure for injection of larvae as outlined under Subheading 3.6.1, but inject only 20 larvae with viral inoculum.
2. Harvest the infected larvae approximately 3.5 days after inoculation.
3. Freeze the larvae at −20 °C.
4. Just before inoculation, weigh the frozen larvae.
5. For each 200 cups with larvae to be inoculated, place 0.5 g frozen larvae and 0.5 mL water in a mortar, and homogenize to a slurry using a pestle.
6. Prepare approximately 700 mL of a solution of blue dye in water, using either 0.5 mg/mL FD&C #1 or ~1 % food coloring dye.
7. Dilute the slurry with dyed water to 660 mL and pour the inoculum through a 100 mesh screen or four layers of cheesecloth to remove large pieces of insect debris. This mixture is the POV inoculum. The remaining 40 mL of dyed water is the negative control inoculum.
8. Apply 1 mL control inoculum to each of three diet-filled cups. Swirl the cups to cover the entire surface with inoculum and let it soak into the diet. Mark these cups as control treatments.
9. In the same manner, apply 1 mL POV inoculum to each of the remaining cups.
10. Using soft tweezers, place 25 late fourth instar *T. ni* larvae onto the treated diet.
11. Inspect the larvae daily and harvest after approximately 4–5 days, when approximately 10 % of them have died from the virus infection. If the larvae are not to be processed immediately, then they should be stored at −80 °C.

### 3.6.3 Larval Transfection

Infection of insect larvae with recombinant viral DNA is the fastest way to express recombinant protein in larvae. It has two advantages over the other methods described in the preceding paragraphs: (a) it eliminates the need to set up and maintain a system of cultured insect cells, and (b) it cuts almost a week off the time between cloning of the heterologous gene and expression of recombinant protein in *T. ni* larvae (*see* **Note 14**).

Since this method is new, we illustrate it using a specific example, the expression under the control of the AcMNPV polyhedrin promoter of DsRed, a red fluorescing protein derived from *Discosoma* coral [31].

1. Amplify by PCR a fragment containing the open reading frame of DsRed from pDsRed (BD Biosciences/Clontech, Palo Alto, CA), adding *Bpl* I restriction enzyme sites on both ends.
2. Digest the resulting fragment of approximately 700 base pairs with *Bpl*I, then treat with Klenow enzyme, digest with *Bgl*II, and ligate into the vector pFastBac1 (Bac-to-Bac, Invitrogen) previously cut with *Bam*HI and *Stu*I. This results in the donor vector pFB1DsRed, with the DsRed gene under the control of the AcMNPV polyhedrin gene promoter.
3. Follow the manufacturer's recommendations for the Bac-to-Bac system to make recombinant Bacmid DNA carrying the DsRed gene in DH10Bac cells.
4. Isolate Bacmid DNA with the aid of the Wizard Plus Minipreps DNA Purification System.
5. Measure the concentration of the DNA in the resulting miniprep and adjust to 1 mg/mL with sterile water.
6. For control inoculum, mix gently 10 μL Cellfectin and 15 μL Sf-900 II medium (Invitrogen) and let sit at room temperature for at least 15 min before use.
7. For transfection inoculum, mix gently 40 μL Cellfectin, 10 μL miniprep DNA (1 mg/mL), and 50 μL Sf-900 II medium and let sit at room temperature for at least 15 min before use.
8. Select approximately 100 early fifth instar *T. ni* larvae as under Subheading 3.4.
9. Inject 20 larvae with 1 μL control inoculum each, and 80 larvae with 1 μL transfection inoculum each, as described under Subheading 3.6.1.
10. Incubate the larvae, and remove any dead larvae after 24 h as described under Subheading 3.6.1.
11. Harvest the infected larvae between 96 and 110 h after inoculation. The success rate of transfection can easily be monitored by the change in color of infected larvae, from pale green to bright red, caused by the fluorescence of DsRed under ambient light.

In our hands the infection rate was 81 % while mortality caused by the injection procedure was 10 % (*see* **Note 15**).

12. Store harvested larvae at −80 °C until processing.

### 3.7 Incubation and Harvest Time

Controlling the incubation conditions of the larvae during infection is very important. Infected larvae are more vulnerable to bacteria and fungi, and it is therefore necessary to allow sufficient air exchange so that no condensation occurs in the containers. However, drying out of the diet should also be avoided.

The time chosen to harvest the infected larvae may be critical for the amount and quality of target protein recovered. Protein synthesis occurs until shortly before the death of the insect, but at the same time protein degradation may also increase towards the end of the infection cycle. The ideal time to harvest is different for each larva, since the course of virus infection and expression of a heterologous protein in a group of larvae are not completely synchronous. For a protein of average stability, we recommend that larvae should be harvested just prior to death: in practice, when ~ 10 % of the population has died.

### 3.8 Homogenization, Clarification, Extraction and Purification

A comprehensive description of protein separation and purification methods is beyond the scope of this work. Typically, homogenization of small batches in an extraction buffer is accomplished with a tissue grinder or a blender, followed by centrifugation to remove non-soluble material and lipids. These steps are carried out while the sample is kept cold, and may include the use of protease inhibitors to prevent in-process degradation. One aspect of downstream processing that differs from cultured insect cell-based systems is worth noting here. Insects maintain a complex pathway in their hemolymph that, when triggered in the presence of oxygen, leads to melanization. A homogenate of larvae will become dark gray to black in a matter of hours, even when refrigerated. Melanization can be prevented by the addition of either 25 μM PTU (1/1000 volume of 25 mM PTU in alcohol) or 5 mM BME.

After the homogenate has been clarified, the target protein may be purified by affinity chromatography or any other suitable method.

## 4 Notes

1. Purchasing from a commercial vendor guarantees a certain level of quality and consistency. It is usually possible to purchase eggs or larvae, and eggs may be delivered either attached to a substrate (e.g., muslin or paper towel) or loose in a container. In either case the eggs should have been surface-sterilized by the vendor. Handling of loose eggs is somewhat more complex than handling eggs on substrate, whereas purchase of larvae is obviously the easiest.

2. USDA APHIS permit 526 is required to house and transport cabbage looper specimens (*T. ni*). For additional information regarding how to apply for your permit visit the following web site: http://www.aphis.usda.gov/plant_health/permits/organism/plantpest_howtoapply.shtml.
3. Several suitable insect diets have been developed for rearing cabbage loopers. These are usually agar-based and contain carbohydrates, plant proteins, vitamins, micronutrients, and antibiotics. For the production of recombinant proteins that are free of mammalian components it may be necessary to ascertain that no animal-derived components are included in the diet.
4. If a type of container is used other than the recommended 8-oz cups, then the number of larvae placed in the cup should be between two and three per $in^2$ diet surface.
5. A balance can also be used to determine the larval development stage. However, measurement of head capsule width under a dissecting microscope is a more reliable method.
6. Using Bacmid DNA (Bac-to-Bac, Invitrogen), transfection of Sf-9 cells has, in our hands, been more efficient than that of Tn-5 cells. Methods for growing insect cell types and for virus titer determination ($TCID_{50}$ or overlay plaque assay) can be found elsewhere in this book (Chapter 9 and Chapters 4, 5, 10, 11 and 22, respectively).
7. Serum-containing and serum-free media are available for these cell lines. Since insect cells are ostensibly free of mammalian pathogens, it may be important under certain circumstances to exclude any possibility of contamination via serum derived from animal sources.
8. Insect hemolymph undergoes a cascade reaction when exposed to oxygen, resulting in the formation of melanin. This "blackening" of hemolymph and the entire insect homogenate should be prevented. Two commonly used inhibitors are β-mercaptoethanol (BME) and phenylthiourea (PTU). BME is also a potent disruptor of protein disulfide bridges, and this function often precludes its use. PTU is extremely toxic, but it is effective even at very low concentrations.
9. Larvae feed continuously except when molting, during which time they will migrate away from the diet. By removing larvae that are feeding, while retaining those that are molting, this behavioral trait can be used to improve developmental homogeneity of the population.
10. Larval development can be slowed (but not increased) by changing the incubation temperature. Larval development essentially stops at 11 °C, and at room temperature development takes approximately twice as long. Note that larval development decreases rapidly at temperatures above 29 °C.

11. With regard to the safety of recombinant baculoviruses, it is important to note that baculoviruses are not infectious to humans. They are classified under Biosafety Level 1, which represents a basic level of containment that relies on standard microbiological practices, with no special primary or secondary barriers recommended other than a sink for handwashing. The NIH classifies experiments with recombinant baculoviruses in the lowest Risk Group, RG1. Material contaminated with recombinant baculovirus should be autoclaved before disposal.
12. Another method that enables in vitro generation of recombinant DNA is direct ligation (Baculodirect™, Invitrogen). We have not tested whether this DNA is suitable for larval transfection.
13. Injection can be done without cooling the larvae; in fact, if one has acquired some experience, it is faster.
14. A similar method has recently been described for the transfection of *B. mori* larvae [32].
15. By injecting fourth-instar instead of fifth-instar larvae, we achieved infection in over 90 % of the larvae. However, due to their smaller size, injecting these larvae was more tedious and led to significantly higher control mortality.

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# Chapter 14

# Production of Virus-Like Particles for Vaccination

**Christine M. Thompson, Marc G. Aucoin, and Amine A. Kamen**

## Abstract

The ability to make a large variety of virus-like particles (VLPs) has been successfully achieved in the baculovirus expression vector system (BEVS)/insect cell system. The production and scale-up of these particles, which are mostly sought as vaccine candidates, are currently being addressed. Furthermore, these VLPs are being investigated as delivery agents for use as therapeutics. The use of host insect cells allows mass production of VLPs in a proven scalable system.

**Key words** Viral vector, Virus-like particle, VLP, Baculovirus, Insect cells

## 1 Introduction

Virus-like particles (VLPs) produced in insect cells have been the subject of research for nearly two decades for their potential use as vaccines. VLPs are structures that form as a result of the simple expression of viral structural proteins and resemble naturally occurring viruses without the nucleic acid content. These particles cannot self-replicate, making them ideal candidates as antigens or immunogens. The baculovirus expression vector system (BEVS) used with host insect cells can produce high levels of recombinant proteins and can perform most of the post-translational modifications of mammalian cells (*see* Chapter 18), thereby retaining the biological activity of the original protein; thus, it is natural to consider this system for the production of these particles. The BEVS is also very efficient at producing large quantities of VLPs and an increasing body of work focusing on the production and the process behind making VLPs has started to accumulate [1–3]. To briefly highlight, this includes work on bluetongue virus [4, 5], rotavirus [6–10], human [5, 11, 12] and porcine [13–16] parvoviruses, human immunodeficiency virus [17, 18], infectious bursal disease virus [19, 20], influenza virus [21, 22], and ebola virus [23].

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VLPs can be composed of either a single or multiple virus proteins. VLPs composed of more than one structural protein can be produced using multiple baculoviruses, each carrying a gene, or with a single baculovirus carrying multiple genes. Sokolenko et al. discuss the benefits and drawbacks that come with working with either platform [24]. VLPs, like the viruses they are modeled after, can be either extracellular or intracellular, i.e., they are secreted into the medium or remain within the cells and must be released through cell lysis, respectively. Additional process considerations and downstream processing accompany the production of secreted VLPs in the BEVS because of the presence of budded recombinant baculovirus, which are often similar in size and morphology to the VLPs [25, 26]. Another important consideration and challenge for producing VLPs in cell culture is to ensure that they do not have any unwanted foreign DNA or RNA trapped inside the particle. One benefit of using the BEVS system to produce VLPs is that DNA from baculovirus and insect cells is either expressed minimally, or not at all, in mammalian systems [27].

Viral vectors produced in insect cells are a natural extension of VLP production. With the incorporation of DNA or RNA having a sequence coding for a transgene of interest, these VLPs gain the potential as a gene therapy agent [28]. Meghrous, Aucoin and their colleagues [29–31] investigated the process behind their production and scaled-up the system to a 20 L bioreactor.

This chapter describes a methodology for producing and monitoring VLPs and viral vectors using the BEVS with a host insect cell based on our experience producing AAV particles and influenza VLPs. These systems will be used as examples.

## 2 Materials

### 2.1 Cell Lines and Recombinant Viruses

1. *Spodoptera frugiperda* cell line Sf-9 (ATCC CRL1711) (*see* **Note 1**).
2. Recombinant *Autographa californa* multiple nucleopolyhedrovirus (AcMNPV) (*see* **Note 2**).

### 2.2 Medium and Solutions

1. Insect cell culture medium (for Sf-9 cells): e.g., 9.5 kg $H_2O$, 384 g Sf-900 II SFM (Gibco® Cell Culture, Invitrogen), 8 mL Sf-900 II Supplement (Gibco® Cell Culture, Invitrogen), adjust pH to 5.9 with sodium hydroxide (NaOH) (10 N); 3.5 g sodium bicarbonate ($NaHCO_3$) solid, then add $H_2O$ to 10 L. Final pH should be $6.2 \pm 0.1$. Adjust pH if necessary with HCl (12 N). Final osmolarity should be $350 \pm 25$ mOsm. Other serum-free media could be used with generally similar growth performances (*see* **Note 3** and Chapter 8).
2. Trypan blue solution (0.4 %).

### 2.3 Shake Flask and Bioreactor Culture

1. Shake flasks, e.g., Erlenmeyer filter top plastic flasks (*see* **Note 4**) (up to 500 mL working volume).
2. Temperature controlled incubator (able to maintain 27 °C, which usually requires a refrigerated incubator) equipped with a shaker table.
3. Bioreactors for larger working volumes, e.g., at mid-scale, a 3.5 L or 22 L Chemap bioreactor (Mannedorf, Switzerland) (*see* **Note 5** and Chapter 11).

### 2.4 Data Acquisition

On-line data acquisition is considered optional, but it can be used to understand the process and to check run reproducibility. A computer with data acquisition hardware/software that can record several signals simultaneously on-line should be installed, i.e., for temperature, DO, pH, gas flow rates, $CO_2$ levels pressure, capacitance, etc. (*see* Chapter 11).

Off-line data acquisition primarily involves determining cell density and viability, e.g., with a hemacytometer (e.g., Hauser Scientific, Horshaw, PA) (*see* **Note 6**).

### 2.5 Post-Harvest

Downstream processing of VLPs should contain several steps that are completely dependent on the type of VLPs produced. The localization of the VLP at harvest time is the most critical factor. For secreted VLPs (e.g., influenza), localization will be in the cell culture supernatant, whereas for intracellular VLPs (e.g., AAV), particles need to be released from the cells prior to further processing. A second consideration regarding downstream processing is the possibility of VLP aggregation occurring during concentration and other downstream processing steps (*see* **Notes 7–10**).

The methods described below can be used for VLP detection. These methods are some of the most commonly used but do come with certain inherent drawbacks (*see* **Note 11**).

#### 2.5.1 SDS-PAGE

1. 1.4 M dithiothreitol (*see* **Note 12**).
2. 5× concentrated tris-glycine running buffer: 800 mL ultrapure $H_2O$ (18.2 MΩ), 15 g Tris, 72 g glycine, 5 g SDS; complete to 1 L with $H_2O$. Store at 4 °C.
3. Sample buffer: 6 mL 0.5 M Tris–HCl, pH 6.8, 5 mL glycerol, 6 mL 20 % w/v SDS, 0.6 mL 4 % bromophenol blue. Store at −20 °C.
4. Mini Protean® II system (Bio-Rad).
5. 4–15 % Tris–HCl Ready Gels (Bio-Rad).

#### 2.5.2 Western Blotting

1. Gel blot paper.
2. Nitrocellulose membrane.
3. PBS (10×): 800 mL $H_2O$, 2 g KCl, 2 g $KH_2PO_4$, 80 g NaCl, 21.6 g $Na_2HPO_4{\cdot}7H_2O$; complete to 1 L with $H_2O$. Filter through a 0.45 μm membrane filter.

4. PBS-T (0.1 %): 100 mL PBS (10×), 900 mL $H_2O$, 1 mL Tween-20.
5. 5 % Dried skim milk in PBS-T (0.1 %): 5 g Blotting grade blocker nonfat dry milk, 100 mL PBS-T (0.1 %); gently heat mixture while stirring until dissolved.
6. Towbin transfer buffer: 700 mL $H_2O$, 3.03 g Tris, 14.41 g glycine, 200 mL methanol; complete to 1 L with $H_2O$.
7. TRANS-BLOT® Semi-Dry Transfer Cell (Bio-Rad).
8. BM Chemiluminescence Blotting Substrate (e.g., Roche Diagnostic Corp).
9. Kodak Image Station.

*2.5.3 Total Protein Analysis*

1. RC DC™ Protein Assay (Bio-Rad) (*see* **Note 13**).
2. UV Spectrophotometer.

*2.5.4 Electron Microscopy*

1. TEN buffer: 10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5.
2. Formvar TEM grid.
3. Centrifuge.
4. 3 % phosphotungstic acid pH 6.0.
5. Electron Microscope.

*2.5.5 Other Equipment*

1. Hot water bath or heating block.

## 3 Methods

### *3.1 Insect Cells*

Verify cell quality prior to amplifying baculovirus stocks and producing VLP.

1. Seed shaker flask with Sf-9 cells at $5 \times 10^5$ cells/mL by diluting with fresh growth medium. Limit the working volume to 20 % of the total flask volume.
2. Determine the viable cell density at least once a day (twice daily would be preferred) until the cell density reaches ~$5 \times 10^6$ cells/mL. Viable and total cells can be counted using a hemacytometer (*see* **Note 6** and Chapter 11). Do not allow the viable cell density to exceed 5–$6 \times 10^6$ cells/mL, i.e., maintain the cells in the exponential growth phase (*see* **Note 14** and Chapter 1) by transferring the appropriate amount of cells to another flask containing fresh medium.
3. Plot the viable cell density vs. time in culture to determine the population doubling time (PDT) (*see* Chapter 1). The PDT should be 24 h or less (i.e., a specific growth rate of ~0.03 $h^{-1}$), which is consistent with healthy cells.

4. If a CEDEX cell counter (or equivalent equipment) is available, then it can be useful to confirm that the cell size distribution is as symmetric as possible and that the cell diameter is consistent with the cell diameter of non-infected cells.

### 3.2 Baculovirus Stock Amplification

Amplify baculovirus stocks using standard methods (e.g., *see* Chapter 11).

### 3.3 VLP and Vector Production

#### 3.3.1 Bioreactor Preparation

Cells that are routinely transferred in fresh serum-free medium and maintained in the exponential growth phase should be used to inoculate the bioreactor at $3–5 \times 10^5$ cells/mL. It is recommended that the data acquisition system be ready to record as soon as the water, used for the sterilization of the bioreactor, is emptied from the bioreactor. The bioreactor should be maintained under a slight positive pressure at all times. Detailed information regarding bioreactor operation can be found in Chapter 11.

1. Add medium, preheated to 27 °C, to the bioreactor.
2. Start the agitation at 110 rpm when using an axial pumping type of impeller such as a helical ribbon impeller or pitch blade impeller.
3. Adjust the DO set point to 30–60 % oxygen saturation (*see* **Note 15**).
4. Once the temperature has stabilized cells can be added to the bioreactor such that the initial cell density is in the range of $3–5 \times 10^5$ cells/mL.

The bioreactor should be operated at its working volume as indicated by the manufacturer to ensure functionality of probes and proper mixing and oxygen transfer through the headspace.

#### 3.3.2 Production Modes

1. Grow the cells to $1.5–2.5 \times 10^6$ cells/mL prior to infecting, allowing the cells to be in the exponential growth phase (*see* Chapter 1).
2. Infect cells at an appropriate MOI (*see* **Note 16**).

Typical dynamics of VLP production of cells infected at an MOI of 5 are shown in Fig. 1.

#### 3.3.3 Culture Harvest and VLP Extraction

The optimal time to harvest VLPs is between 48 and 96 h post-infection (h pi), before significant cell lysis occurs (*see* **Note 17**). The procedure to extract VLP from the cell culture is as follows.

1. Harvest the cells by centrifuging 15 min at $300 \times g$ and 4 °C.
2. Decant supernatant or lyse pellet to remove VLPs in the case where they were not secreted from the cell (*see* **Note 18**).
3. Purify VLPs either by chromatography or a combination of density gradient centrifugation and chromatography methods.

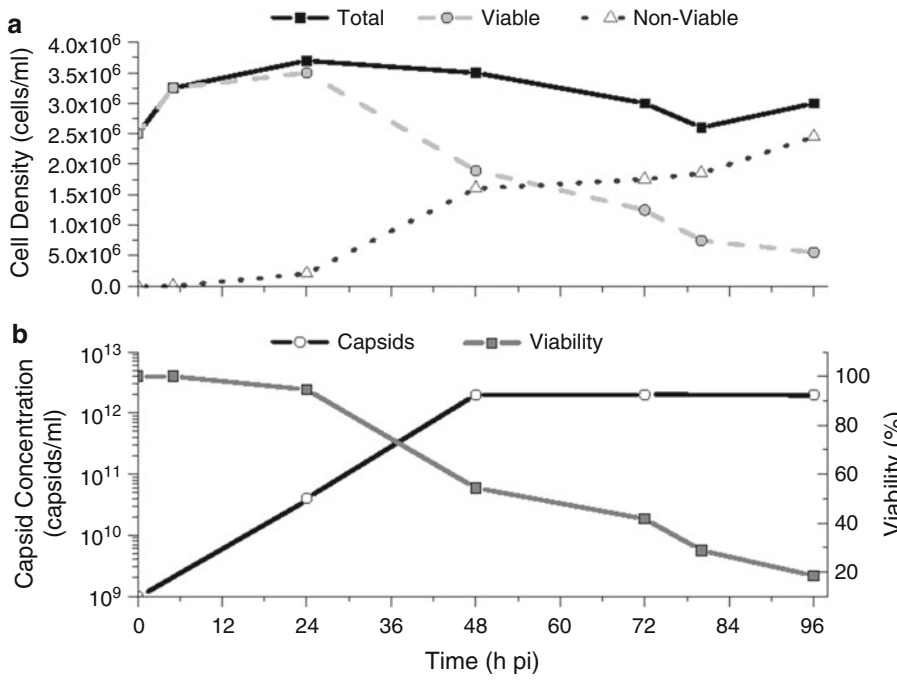


**Fig. 1** Dynamics of adeno-associated virus-like particle production in Sf-9 cells infected at $2.5 \times 10^6$ cells/mL, with BacCap at a MOI of 5 in Sf-900 II medium

*3.3.4 Culture Control*

The following parameters can be controlled and/or monitored during cultivation:

1. Dissolved oxygen concentration.
2. Agitation rate.
3. Temperature (maintained at 27–28 °C).

Product yield can be affected if dissolved oxygen is too high or too low. We routinely use 40 % DO, but it can be anywhere from 30 to 60 % [32]. An example of a controlled bioreactor production is shown in Fig. 2. *See* Chapter 11 for more detail regarding DO control. An increased temperature can be used to increase the gene expression rate and reduce the time to harvest [31]. Other parameters that are not controlled but that are generally monitored include signals that can be collected online or measured offline. On-line parameters that are solely monitored may include signals from a fluorescence probe (e.g., if the GFP reporter protein is produced as discussed in Chapter 22) or a capacitance probe (to measure cell density). Off-line measurements may include cell density, cell viability, VLP concentration, specific protein concentration, and nutrient concentrations (e.g., glucose and glutamine).

*3.3.5 VLP Downstream Processing*

As discussed in **Notes 7–10**, different methods can be used to purify VLPs. After clarification of some VLPs, e.g., influenza or triple layered rotavirus, are concentrated/semi purified with

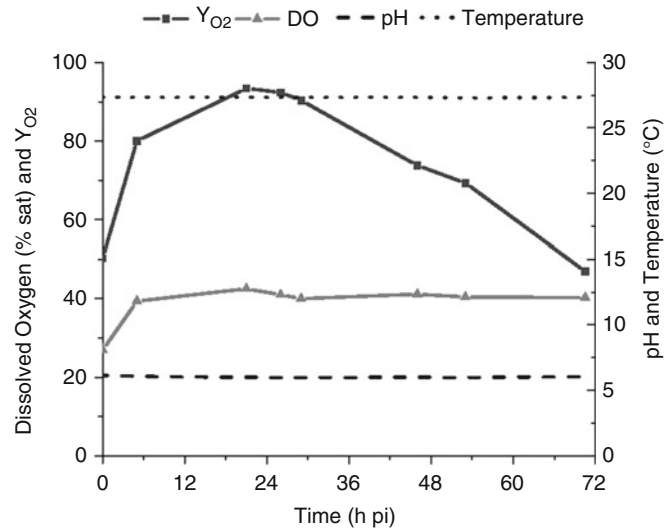


**Fig. 2** Controlled (DO, temperature) and monitored (pH) viral stock production of recombinant baculovirus expressing influenza Neuraminidase protein (NA) in a 3.5 L bioreactor with Sf-9 cells at an MOI of 0.2

ultracentrifugation [22, 33, 34], while others, e.g., parvovirus, can be precipitated [15]. Additives such as protease inhibitors, nucleases and chelating agents may be added (*see* **Note 19**) during these steps or during production. Finally, VLPs must undergo a polishing step in order to be considered clinical grade material, which can be done using chromatography steps based on affinity or ion-exchange mechanisms [1].

### 3.4 VLP Detection

To assess the composition of the particles, individual viral proteins can be assessed using specific antibodies (*see* **Note 20**).

#### 3.4.1 SDS PAGE/Western Analysis

1. Subject cell samples to three freeze/thaw cycles.
2. The quantity of proteins in the samples can be determined using the Bradford method (RC DC™ is used here). 450–600 ng of protein should be loaded for detection.
3. Add 36 µL dithiothreitol (DTT) to 300 µL of sample buffer.
4. Dilute culture samples in sample buffer with reducing agent (*see* **Note 21**).
5. Heat samples in a boiling water bath for 5 min.
6. Centrifuge samples at 16,000 × *g* for 2 min.
7. Load supernatant on gel.
8. Run gel at 200 V for 60 min.
9. Stain with a Coomassie blue or silver stain solution (*see* **Note 22**) or continue to **step 10** for Western blot analysis.

10. Rinse gel in transfer buffer (keep the equilibration time short to prevent diffusion of low molecular weight proteins out of the gel) by changing the buffer three times in 5 min.
11. Prior to the end of the electrophoresis run, soak the blot paper and nitrocellulose (NC) membrane in one container with Towbin Transfer buffer for 15–30 min (keep it at 4 °C), in the following order: (a) 2× Gel blot Paper, (b) 1× nitrocellulose membrane (NC) and (c) 2× Gel blot Paper.
12. Place in the following order, on top of the anode of the TRANS-BLOT Semi-Dry Transfer Cell. Carefully roll out bubbles with a test tube after each layer is laid down (except on the gel): (a) 2× Gel blot paper, (b) 1× NC membrane, (c) gel (avoid moving the gel against the NC membrane once it is laid down) and (d) 2× Gel blot Paper.
13. Carefully place the cathode assembly onto the stack.
14. Run at 10 V for 60 min.
15. Once the transfer is complete remove the NC membrane and dry for 5 min.
16. Block in 5 % dried skim milk/PBS-T (0.1 %) for 1 h at room temperature with shaking.
17. Wash the membrane with PBS-T (0.1 %): (a) 1 × 15 min and (b) 2 × 5 min.
18. Add monoclonal against the desired protein, typically diluted 1:1000 in PBS-T (0.1 %).
19. Incubate overnight with shaking.
20. Wash the NC membrane with PBS-T (0.1 %) with shaking: (a) 1 × 15 min and (b) 2 × 5 min.
21. Add the conjugated secondary antibody in PBS-T (0.1 %) and incubate for 1 h at room temperature with shaking.
22. Wash the NC membrane with PBS-T (0.1 %) with shaking: (a) 1 × 15 min and (b) 4 × 5 min.
23. Detect using BM Chemiluminescence Blotting Substrate and analyze using a Kodak Image Station.

### 3.4.2 Total Protein

The following is a brief description of the standard assay protocol for 5 mL samples from Bio-Rad (*see* **Note 23**).

1. Add 20 μL of DC reagent S to each 1 mL of DC reagent A. This solution is now called Reagent A.
2. Prepare 3–5 dilutions of a protein standard from 0.2 to 1.5 mg/mL protein. This should be done each time the assay is conducted.
3. Pipet 100 μL of standards and samples into clean and dry tubes.
4. Add 500 μL RC reagent I, vortex, incubate for 1 min at room temperature.

5. Add 500 μL RC reagent II, centrifuge tubes at 15,000 × *g* for 3–5 min.
6. Discard supernatant.
7. Add 510 μL reagent A to each tube, vortex then incubate at room temperature for 5 min.
8. Add 4 mL of DC reagent B to each tube, vortex then incubate at room temperature for 15 min.
9. Determine the absorbance at 750 nm.

#### 3.4.3 Negative Staining Electron Microscopy

For additional details *see* **Note 24**.

1. Dilute samples with TEN buffer.
2. Place aliquoted diluted samples in 240 μL microtubes with Formvar and carbon coated grids inserted at the bottom of the tube.
3. Centrifuge tubes at 20,000 × *g* for 5 min.
4. Dry recovered grids and stain with 3 % phosphotungstic acid, pH 6.0.
5. Visualize samples under transmission electron microscope.

## 4 Notes

1. VLP production can be done using Sf-9 or Tn-5 cells [22]. We believe that a better baculovirus stock is produced in Sf-9 cells; however, the total VLP titers (and viral vectors) have been observed to be comparable [35]. Protein Sciences Corporation has developed a proprietary cell line, expresSF+, with the same large size of Tn-5 cells that also possesses the ability to grow to the same high cell densities as Sf-9 cells ($10^8$ cells/mL). This cell line has great potential for VLP production.
2. Recombinant baculoviruses can be produced with a number of different commercially available systems. Invitrogen, Clonetech and BD Biosciences have easy to use systems based on direct cloning into provided vectors and recombination with baculovirus DNA or bacmid production for recombinant virus generation (Bac-to-Bac, BaculoDirect, BacPAK and BaculoGold). The most widely used promoter is polyhedron (polh), however, other promoters, e.g., p10 can also be used. Another consideration is the use of polycistronic viruses for simultaneous expression of proteins of interest, or individual monocistronic viruses coding for each protein for a higher degree of flexibility for final VLP composition [24]. Additional information about generating recombinant baculoviruses can be found in Chapters 3 and 4.

3. Other serum-free media that have been used for VLP production, e.g., HyClone-SFX [36] and IPL-41 [22]. *See* Chapter 8 for more information regarding serum-free media.
4. Our lab routinely uses disposable plastic Erlenmeyer flasks with a vented top. Glass flasks can be used as well, but it is advisable to consistently use the same flask type when cultivating and maintaining cells.
5. Due to the shear sensitivity of the cells, the bioreactor should be equipped with a low shear impeller to ensure adequate mixing. In our lab, the use of a helical ribbon impeller or pitch blade impeller is common and depends solely on the configuration of the reactor and on the probes that are used with the bioreactor. A dissolved oxygen (DO) probe, pH probe, and temperature probe can be installed in the bioreactor to monitor the DO concentration, pH and temperature, respectively. The resulting information can then be used to control these parameters. In addition, a capacitance probe may be installed in the bioreactor to monitor biomass levels. The DO concentration can be controlled either through the use of mass flowmeters (i.e., by controlling air, oxygen and/or nitrogen flowrates) or by varying the agitation and sparging rates. *See* Chapter 11 for information regarding large scale production.
6. The CEDEX automated cell counter (Innovatis, Germany) using the established Trypan blue exclusion method (*see* Chapter 11) can also be used here. In addition to cell concentration, the CEDEX records viability, average cell diameter and has a useful multi-sampler application.
7. Regardless of whether the VLPs are intracellular or secreted, the first step is to separate the supernatant from the cells directly after harvest. This is traditionally done with centrifugation, although filtration can also be used. In the case of intracellular VLPs, the cells need to be lysed to release VLPs. During primary recovery, VLPs are at the highest risk of aggregating, either with themselves or with cellular debris (e.g., proteins and nucleic acids) left in the culture medium. Chahal et al. [37] investigated different lysing techniques (freeze-thaw, surfactant and two phase extraction), aggregation prevention methods (salt addition, nuclease treatment) and resuspension buffers for AAV vectors. In the case of influenza VLPs, aggregation may also prove to be a problem during exploration of novel bioprocessing steps intended to improve VLP final concentration.
8. After primary recovery, the next step is concentration and/or intermediate purification. This step can utilize ultracentrifugation, ultrafiltration, or diafiltration and should aim to separate VLPs from free proteins. When VLPs are concentrated by ultracentrifugation, different speeds and times have been

used, e.g., 130,000–200,000 × *g* for 90 min [22, 33] and 27,000–37,000 × *g* with and without a 25 % sucrose cushion [26]. In our lab we use a 25 % sucrose cushion and a centrifugation time of 3 h at 37,000 × *g* in a Sorvall Discovery SE 100 ultracentrifuge (Thermo Fisher Scientific, USA). VLPs are then resuspended in another buffer (PBS, Tris–HCl, or HEPES), but studies to determine which buffer is most appropriate for VLP stability still need to be conducted. In the case of diafiltration/ultrafiltration we use a 100 k MW cut off centrifugal filter (PALL Corporation, USA) and follow the guidelines given in the specification booklet for centrifugation speed and time (e.g., for 4 mL filter units, 4000 × *g* for 20 min concentrates 10–20× influenza VLPs). Using molecular weight cutoff filters are fast and convenient for concentrating smaller volumes (i.e., 4–20 mL filter units).

9. The polishing step to produce clinical grade material can be done using another density gradient ultracentrifugation with sucrose, cesium chloride, or iodixanol, commercially known as Optiprep (Nycomed, Norway). Alternatively, chromatography can be used.
10. Another aspect to take into consideration when producing secreted VLPs is recombinant baculovirus contamination. Both particles can be similar in size and this can complicate downstream processing. See the review by Vicente et al. [1] for more information on downstream processing of VLPs for vaccines.
11. Other methods are currently under intense development for VLP detection and quantification. For example, for influenza VLPs, such methods include kinetic monitoring of secreted VLP production using Fluorescence-Activated Cell Sorting (FACS), and identification of total VLPs and HA by HPLC. Additionally, for influenza VLPs, other methods currently used for whole virus quantification, e.g., the hemagglutination assay or Single Radial Immunodiffusion (SRID) assay, can be used to determine the antigenic activity of the VLPs. While EM and total protein are methods that can be used to verify the presence of VLPs and quantify their total protein content, these methods have drawbacks, including a need for high purity for accurate analysis, thereby making them too laborious for process development. For influenza VLPs, the major drawbacks in using western blots and ELISA are antibody specificity variability and the lack of verified standards. An anti-HA antibody for one strain of influenza may or may not work for other strains, thereby resulting in a wide variety of antibodies to choose from and validate. For AAV quantification and detection, ELISA used to analyze the VLP, while transduction and infection based assays are used to analyze the

activity of the vector form of the particles. *See* Aucoin et al. [38] for a summary of currently available methods.

12. B-mercaptoethanol can also be used.
13. Other Bradford assays could be used, such as the Quick start Bradford, the Bio-Rad protein assay, or the DC protein assay. The Quick start and Bio-Rad assays are compatible with samples containing reducing agent and not with those containing detergents. The DC assay is compatible with samples containing detergents but not reducing agents. The RC DC™ method, however, is compatible with both. This assay has been chosen in our lab out of convenience because it can be used with samples from lysed cells that may contain traces of detergent or with the same samples prepared for SDS electrophoresis. All assays work in the range from 0.2 to 1.5 mg/mL.
14. Cells can be passaged either by inoculating the old cells into fresh medium or by centrifuging cells and resuspending them in fresh medium to achieve the desired concentration of $5 \times 10^5$ cells/mL. While completely replacing the medium will ensure that all the toxins are removed, we have found in our experience that inoculation of the old cells into fresh medium is an adequate technique and have not encountered limited cell growth due to existing toxins. However, we do not passage cells at cell densities higher than $5–6 \times 10^6$ and require a viability of at least 95 %. If cells have been grown to a higher concentration and have a lower viability, then complete medium replacement is generally needed.
15. For shake flasks, loosening the lid and maintaining a low working volume to flask volume ratio (which provides a large liquid–gas interface to volume ratio) results in good oxygen transfer. In a bioreactor the agitation rate is maintained constant for the entire batch to maintain ~constant mass transfer rates. Insect cells do not have a cell wall and are therefore more susceptible to physical damage from hydrodynamic shear stress and bubble formation. Some investigators have used agitation speed as a means to control the DO concentration and have found no adverse effect on the cells until a shear stress of between 1.5 and 4 $N/m^2$ is reached, at which point viability decreases. However, it has been shown that cell growth is affected by shear stress before cell death occurs. Therefore, there is an optimal agitation/aeration rate that ensures adequate DO concentration and allows cells to grow and produce optimal amounts of VLPs [18]. By keeping the agitation rate constant, the mass transfer coefficient, $k_La$, can be assumed to be approximately constant.
16. High MOIs lead to a synchronous infection and faster production, often with the highest yields. This has to be balanced, however, by the volume of virus stock that has to be supplied

to achieve the high MOIs. A rule of thumb is to avoid infecting a cell culture with a virus stock volume greater than 10 % of the cell culture volume. Infection at higher cell densities can be done if cells are infected in fresh medium under non-limiting nutrient conditions or under a fed batch mode to increase yields. The advantage of changing medium prior to infection is to ensure that limiting nutrients are replenished and inhibitory compounds are eliminated from the cell culture during the production phase; however, there are significant costs associated with replenishing the entire medium as well as potential damage to the cells through the manipulations involved with removing the medium from the cells. At large scale this step may become difficult without appropriate equipment and the chances of contaminating the culture increase. In some cases, concentration of the virus stocks may be necessary [39]. For more details *see* Chapter 11.

17. The harvest time depends on the downstream processing methodology. If it is desired to keep the VLP associated with the cell, i.e., the VLP is intracellular and loss is to be minimized by avoiding cell lysis, then the harvest should be done by 72 h pi. However, by leaving the culture up to 96 h pi, and treating both the supernatant and the pellet, the process is more robust with respect to the initial MOIs of each baculovirus used.
18. The best method to lyse cells depends upon the scale of operation. Cells are generally lysed after the clarification step. At small scale, freeze-thaw cycles prove to be the best method for product recovery, but are not appropriate at larger scales. Chahal et al. [35] discuss different methods of lysing cells, as well as buffer selection in the case of intracellular VLPs while taking scale into consideration. For secreted influenza VLPs, lysing the cells is not done and particle concentration is usually required prior to conducting additional purification steps. After ultracentrifugation or during ultrafiltration, the VLPs are resuspended in another buffer (PBS, Tris–HCl or other). In theory, this can be advantageous because it removes potentially harmful cell debris that could be left over after clarification; however, to our knowledge no stability studies in different buffers have been completed to verify this assumption. For small volumes (<20 mL), ultrafiltration is the fastest (about 20–45 min to spin down 10×), while the pelleting takes 3 h itself, not including prep time.
19. Due to the amount of host cell proteases, it is advisable to add protease inhibitors during the early steps of downstream processing [1]. Additionally, for secreted VLPs, nucleases can be added before harvest or after clarification to dissociate the VLPs from DNA released by lysed cells. Chelating agents have been shown to have an effect on the stability of SV40 capsid

VLPs [40], i.e., promoting capsid dissociation. In the case of intracellular VLPs, capsid dissociation may be encouraged to remove foreign DNA trapped inside VLPs. In this case the VLPs need to reassemble after DNA removal. All of these agents can be removed in later purification steps. For protease inhibitors and nucleases, this can be accomplished with chromatography or ultracentrifugation, while for chelating agents, such as EDTA, this can be accomplished with a buffer exchange. For protease inhibitors and nuclease detection, Abcam (Cambridge, MA) and Merck KGaA (Darmstadt, Germany) have commercial ELISA kits available.

20. For AAV detection, monoclonal antibodies for Rep 78, 68, 52 and 40 proteins and VP 1, 2 and 3 are used (Maine Biotechnology Services, Portland, ME). For influenza proteins, polyclonal sheep antibodies against HA (detects HA1 and HA0) and NA (NIBSC, Hertfordshire, UK) and a monoclonal mouse antibody for M1 are used (Abcam, Cambridge, MA). For influenza, antibodies were chosen based on their activity with an in-house wild type virus, level of background and cost. Another antibody that was used for HA is a mouse monoclonal anti-HA that detects HA2 and HA0 (Santa Cruz Biotechnology, Santa Cruz, CA), but resulted in a varying signal in our hands (results unpublished). The advantage of monoclonal antibodies over polyclonal antibodies is their specificity, which results in low background noise, while the advantage for polyclonal antibodies is lower cost. When analyzing crude samples, it is important to use an antibody that generates the lowest possible background noise to clearly detect VLP proteins. Background noise can also come from secondary antibodies, so it is advisable to run a control with only the secondary antibody to know what kind of background signals it produces. While laborious, it is best to probe with antibodies for all of the antigens that the VLP is composed of to get an idea of the levels of each in the particles. Another consideration for antibody choice is the strain of virus being used to model VLPs. This is important for influenza VLPs because the virus is classified into three subtypes (A, B, and C) with each containing different strains due to the high mutation rate of the surface proteins (HA and NA). Therefore, one antibody for a strain within subtype A may not be suitable for another in subtype A. There has been some work on developing universal antibodies for influenza focusing on a conserved region of the HA protein that may be of interest in the future to solve this problem of strain-to-strain variability [41].

21. When reducing samples with DTT, we use a mixture of 2 parts sample to 1 part sample buffer with DTT (to give a final DTT concentration of 50 mM).

22. Coomassie blue is able to detect as low as 50 ng of protein; however, silver stain is 50× more sensitive. Therefore, silver stain will not only allow the visualization of the protein of interest, but can also identify contaminant proteins that may be present at low levels.
23. The total protein assay is used as a method to determine the amount of loading material for gels and also to analyze protein content during all stages of the process. Bradford protein assays are robust and relatively cheap ways to gain insight into variations of the total protein content during downstream processing steps. This is important for influenza VLPs because as to date there is no proven standardized method to process and quantify. With all these unknown variables coming into play at once, using a universally known and robust method to quantify total protein at all stages of process, and method development, is a valuable tool can that take one unknown variable out of the equation.
24. The method described here has been used in our lab for previous virus samples and could also be used for VLPs. It has been adapted from Alain et al. [42]. NSEM provides a method to physically quantify total particles and is usually done after the second purification step, i.e., density gradient ultracentrifugation, on fractions containing VLPs identified from Western blotting. It can be done earlier, but VLPs may be harder to visualize with remaining contaminants. A concentration of $10^5$–$10^6$ virus particles per mL is typically needed for visualization [43].

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# Chapter 15

# Alternative Strategies for Expressing Multicomponent Protein Complexes in Insect Cells

**Stephanie Chen**

## Abstract

Expression of recombinant proteins in insect cells infected with baculoviruses is commonplace. This system provides an easy way to generate a significant amount of properly folded, functional protein with proper posttranslational modifications and can be used to effectively produce multi-protein complexes. This chapter describes an alternative method of expressing high order protein complexes in insect cell culture. Specific examples involving the expression of 5- and 8-protein complexes are discussed.

**Key words** Baculovirus infected insect cells, BIIC, *Spodoptera frugiperda*, Sf-9, Baculovirus, Multicomponent protein, Co-expression, Recombinant co-expression

## 1 Introduction

The scientific world's search for disease relevant targets has increasingly focused on more complex multicomponent proteins. Mega-dalton protein complexes are currently drug targets for the treatment of cancer (e.g., proteasome inhibitor—bortezomib) and bacterial infections (e.g., ribosome inhibitor—chloramphenicol). These protein complexes are relatively large and stable, but there are a large number of smaller complexes that can be very dynamic in their interactions. Many enzymes are nonfunctional in isolation, but become catalytically viable upon binding to their partner proteins [1]. In addition, protein function is highly regulated in the cell, where association with different partners can lead to different substrate specificity or subcellular localization [2]. This level of regulation is critical to understand as drug development efforts proceed. In addition, many potential drug targets can exist in protein complexes that facilitate catalytic cascades by the physical interaction of multiple enzymes (e.g., bacterial Acetyl Coenzyme A Carboxylase).

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The challenge is to find a robust method to express these complex proteins. Expression of recombinant proteins in insect cells by infecting with recombinant baculoviruses is commonly used to produce proteins from higher order organisms. This approach offers the ability to generate a significant amount of properly folded, functional protein with proper posttranslational modifications. Furthermore, it is fast, easy, and less costly than mammalian systems, and offers the advantage of co-expressing multiple proteins. The use of preinfected cells (referred to as baculovirus infected insect cells (BIIC)) significantly improves consistency in protein production without the need for generating a stable system. Briefly, this process involves (1) generating a recombinant baculovirus for the protein to be expressed, (2) infecting insect cells with this recombinant baculovirus, (3) concentrating and freezing the infected cell suspension (i.e., BIIC), and (4) thawing the BIIC for the protein to be expressed and infecting insect cells to produce the desired protein. The use of BIIC with the *Spodoptera frugiperda* Sf-9 insect cell line has greatly advanced the ability to generate both single and multicomponent proteins [3, 4]. Storage of BIIC in liquid nitrogen decreases storage space and improves virus stability. Easy, efficient storage allows for consistent expression results since a single batch of BIIC can be generated to meet a wide range of scale up volumes. The traditional approach of using the baculovirus system to produce recombinant proteins, i.e., adding a baculovirus suspension to an insect cell culture in exponential growth phase, is limited by the total volume of virus (and therefore spent medium) that can be added to the culture without compromising cell growth and viability. In contrast, expression with BIIC requires very little volume added per component with no addition of spent medium. In addition, having each component generated independently (as opposed to cloning 2 or more genes onto a single expression plasmid) allows for maximum flexibility in determining the optimal tagging strategy (for purifying complex) and the optimal virus ratio to obtain the best purified protein complex.

This method has been used to successfully generate dimeric and trimeric proteins [5]. This has been taken another step forward and separately generated baculovirus stocks were used to co-express five and eight component protein complexes. These full complexes can be purified by affinity chromatography when a single subunit has been tagged and co-expressed with the remaining untagged subunits. In addition, it has also been shown that changing the tagged subunit can impact expression and yield and that using different ratios of BIIC for infection of the components can alter yields and activity.

## 2 Materials

### 2.1 Cell Culture

1. Sf-9 (*Spodoptera frugiperda*) cells (*see* **Note 1**).
2. Sterile culture flasks.
3. Sterile serological pipettes.
4. Sterile conical tubes of various sizes (15–500 mL).
5. Shaking incubator.
6. Serum-free insect cell medium, e.g., HyClone® SFX-Insect (*see* Chapter 8 for other media).
7. Heat-inactivated fetal bovine serum (FBS).
8. Vi-Cell® (Beckman Coulter, Inc.) or other cell counter with the ability to measure average cell diameter.
9. Vi-Cell® sample cups.
10. Baculoviruses (P0) generated for expression of all desired proteins.

### 2.2 Cryopreservation

1. Freezing medium: 20 % (v/v) heat-inactivated FBS, 10 % DMSO, 70 % culture medium.
2. 2 mL sterile cryo vials.
3. Mr. Frosty (NALGENE®) rate controlled freezer or similar controlled freezing device.

### 2.3 Automated Purification

1. Branson Sonifier 450 or equivalent.
2. Eppendorf Centrifuge 5415C or equivalent.
3. KingFisher™ mL Magnetic Particle Processor.
4. Promega MagnaHis™ Ni-Particles (Promega) or Anti-FLAG® M2 magnetic beads (Sigma).
5. Appropriate KingFisher™ mL plasticware (sample cups, sleeve covers, etc.).
6. Lysis buffer for MagnaHis™ or Anti-FLAG® M2 purification: 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, 0.05 % (v/v) Tween 20, pH 8.0. For *E. coli* lysis, add 1 mg/mL lysozyme.
7. Wash buffer for MagnaHis™ or Anti-FLAG® M2 purification: 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 0.05 % (v/v) Tween 20, pH 8.0.
8. Elution buffer for MagnaHis™ purification: 50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, 0.05 % (v/v) Tween 20, pH 8.0.
9. Elution buffer for Anti-FLAG® M2 purification: Tris Buffered Saline (TBS) pH 7.5, 0.1 mg/mL FLAG® peptide.

### 2.4 Gel Analysis

1. Pre-prepared 4–12 % SDS-PAGE gels.
2. MOPS or MES SDS running buffer.
3. Gel running apparatus.
4. Power source.
5. Protein gel stain.
6. Protein gel standard.

## 3 Methods

### 3.1 Preparation of Baculovirus Infected Insect Cells (BIIC)

BIIC should be prepared separately for each complex component protein to be expressed.

1. Split Sf-9 cells the day before infection such that they are in exponential growth phase at the time of infection. Our Sf-9 cells double about every 20 h and are in the exponential growth phase at $1–5 \times 10^6$ cells/mL (this is medium-dependent). We infect the cells at ~$2 \times 10^6$ cells/mL.
2. Within 24 h after splitting the cells (**step 1**), infect cells with the desired P0 baculovirus (BV) at a 1:200 ratio (mL baculovirus suspension–mL cell culture). We infect a 200 mL cell culture with 1 mL baculovirus suspension.
   (a) Note cell density, viability, and average cell diameter at the time of infection.
   (b) Viability should be at least 90–95 %.
   (c) Add 5 % heat-inactivated FBS at time of infection.
3. Incubate culture at 27 °C and 130 rpm overnight.
4. On the next day determine the cell density, cell viability and average cell diameter. Continue monitoring these parameters until the average cell diameter has increased by 15–25 % (ideally, 20 %; our cell diameter typically shifts from ~15 to ~18 μm). The cells should still have a good viability (90–95 %); if not, then repeat the infection process beginning with **step 1**.
5. Remove $10^8$ cells and centrifuge at $1000 \times g$ for 5–10 min.
6. Pour off supernatant in sterile hood, and then suspend the pellet into 10 mL cold freezing medium.
7. Aliquot the resulting cell suspension into cryovials at $10^7$ cells/vial, i.e., 1 mL cell suspension/vial.
8. Freeze the vials using Mr. Frosty (or equivalent) by following vendor's instructions.
9. Transfer to liquid nitrogen for long term storage.

### 3.2 Expression of Multicomponent Protein Complexes in Sf-9 Cells with BIIC

*See* **Note 2** for additional information.

1. Split Sf-9 cells the day before infection such that they are in early exponential growth phase at the time of infection. We split cells so they are 1.3–1.6 × $10^6$ cells/mL at the time of infection.
2. At the time of infection, note the viable cell count, viability, and average cell diameter.
3. Thaw 1 vial of BIIC for each protein component to be co-expressed.
4. Add $10^6$ thawed BIIC cells for each component of the multi-component protein complex per each liter of culture (i.e., 1 vial of BIIC prepared as above per component per 10 L culture).
5. Incubate culture at 27 °C. 3 L flasks should be incubated at 90 rpm; smaller flasks should be incubated at 130 rpm.
6. Determine the viable cell count, viability, and average cell diameter daily.
7. By ~48 h post-infection (pi), cell growth should stop, the viability should still be ≥92 %, and there should be a 15–25 % increase in average cell diameter. If the viability drops below 75 %, then harvest cell paste as indicated in **step 8**.
8. At 66–72 h pi determine the viable cell count, viability, and average cell diameter. Viability should be 85–95 %. Harvest the cell paste by centrifuging the culture at 1140–1780 × *g* for 5–10 min. Centrifuge ~5 mL of culture separately to use for analysis.

### 3.3 Expression Analysis

1. Resuspend pellet from 5 mL of culture (from **step 8** of Subheading 3.2) in 1 mL BioSprint lysis buffer + 0.1 % Tween 20.
2. Sonicate the resuspended culture at full power for 1 min on ice.
3. Remove 120 μL of crude lysate from **step 2** and transfer to a clean Eppendorf tube.
4. Centrifuge remaining crude lysate (~1 mL) and 120 μL sample from **step 3** at 16,000 × *g* in a microfuge for 10 min to separate particulate ("insoluble") matter.
5. Transfer supernatant ("soluble") fraction from 120 μL sample to a clean Eppendorf tube.
6. Resuspend the pellet from the 120 μL sample ("insoluble") in 120 μL BioSprint lysis buffer + 0.1 % Tween 20.

### 3.4 KingFisher mL Automated Purification

*See* **Note 3** for additional information.

1. Transfer the supernatant from the ~1 mL sample (from Subheading 3.3) to a KingFisher mL sample cup.
2. Add 50 μL Promega MagnaHis™ or 100 μL Anti-FLAG® M2 magnetic beads resin to the sample.

3. Prepare remaining sample cups with appropriate wash buffer and elution buffer. We typically elute two times with 150 μL elution buffer (300 μL total).
4. Use the MagnaHis™ Protocol for purification (*see* manufacture's protocol).
5. Pool eluates when protocol is complete.

### 3.5 Gel Analysis

*See* **Note 4** for additional information.

1. Run insoluble lysate, soluble lysate, and BioSprint eluant samples on an SDS-PAGE gel with appropriate protein standards.
2. Stain gel and visualize.

### 3.6 Example 1—PRC2

PRC2 is a five member complex composed of EZH2, EED, SUZ12, AEBP2, and RbAp48. EZH2 is the functional methyltransferase domain for which activity was measured to determine the quality of purified protein. Each subunit was cloned either untagged or with an N-terminal FLAG® tag. To determine which of the five subunits should be tagged for optimal production/purification of the full complex, five 800 mL cultures of Sf-9 cells were grown in 3 L shake flasks. Each 800 mL culture was infected with $1.6 \times 10^6$ BIIC generated for each subunit (EZH2, EED, SUZ12, AEBP2, RbAp48). For each of the five flasks, only one subunit was tagged with an N-terminal FLAG® tag. This tagged subunit was co-expressed with the four remaining untagged subunits. Our goal was to achieve a PRC2 complex with a molar stoichiometry of 1:1:1:1:1 (EZH2–EED–SUZ12–AEBP2–RbAp48). However, purification using overexpression of a single tagged subunit yields an overabundance of that subunit compared to the untagged members of the complex. To determine if we could obtain a more optimal stoichiometry, 800 mL of Sf-9 cells grown in a 3 L shake flask were infected with $0.8 \times 10^6$ BIIC generated for expression of Flag-EZH2 (0.5 V) + $1.6 \times 10^6$ BIIC generated for each of the remaining untagged subunits. The complex from all cultures were purified with Anti-FLAG® M2 resin, dialyzed to remove the FLAG® peptide, and concentrated. Gel analysis demonstrated that each subunit can be detected by SDS-PAGE for all samples (Fig. 1). PRC2 expressed and purified using the FLAG-EZH2 tagged subunit produced a complex closest to the desired 1:1:1:1:1 stoichiometry. Assay data indicated that the activity of the complex purified with tagged EZH2 was significantly higher than that seen with any of the complexes purified when any of the other subunits were tagged (Fig. 2). PRC2 expressed with 0.5 V FLAG-EZH2 BIIC was more pure and had a higher specific activity, but yields were much lower than the sample purified from PRC2 expressed with an equal volume of FLAG-EZH2 BIIC (Figs 1 and 2). It is speculated that the high specific activity obtained with the 0.5 V FLAG-EZH2 BIIC is due to an improvement in the stoichiometry of the components of the complex [6].

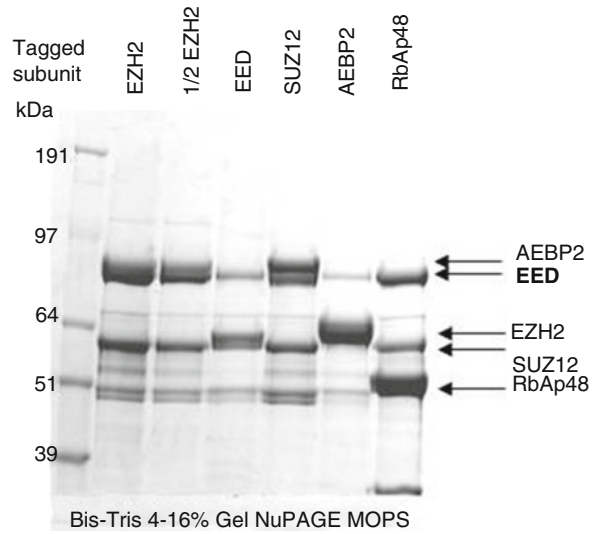


**Fig. 1** SDS-PAGE analysis of PRC2 expression with individually tagged complex members and expression using FLAG-EZH2 at 0.5 V and 1.0 V BIIC for remaining complex members. PRC2 purified using the FLAG-EZH2 tagged subunit resulted in a complex closest to the desired molar stoichiometry for that expressed with either 1.0 V or 0.5 V BIIC. PRC2 purified from the culture infected with 0.5 V FLAG-EZH2 BIIC was the most pure, but had the lowest protein yields

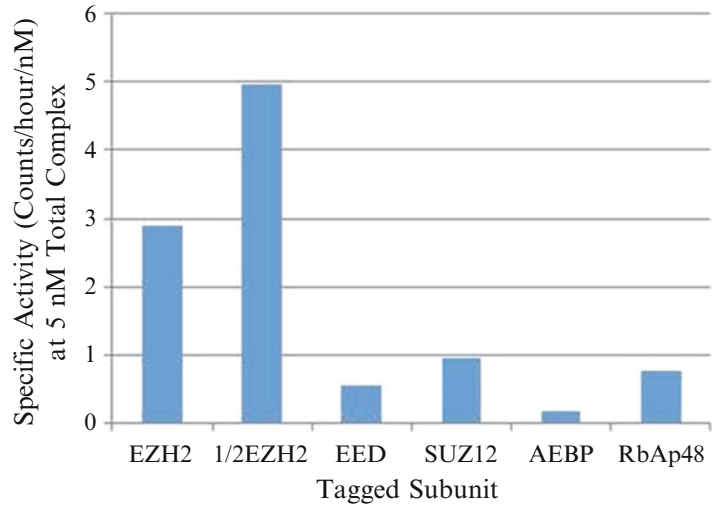


**Fig. 2** SPA assay analysis of EZH2 activity for samples from purified PRC2 expressed with individually tagged complex members and using FLAG-EZH2 at 0.5 V and 1.0 V BIIC for the remaining complex members. The complex purified using the FLAG-EZH2 tagged subunit is higher in EZH2 activity than that purified using any other tagged subunit. Activity of the complex generated using 0.5 V FLAG-EZH2 BIIC has a highest EZH2 activity than the complex generated using 1.0 V FLAG-EZH2 BIIC

### 3.7 Example 2—CSN (COP9 Signalosome)

CSN is an eight member protein complex composed of COPS1, COPS2, COPS3, COPS4, COPS5, COPS6, COPS7a and b (only one isoform present in the complex, but both used in expression work), and COPS8. COPS5 is the functional deneddylase domain for which activity was measured to determine the quality of purified protein. Each subunit was cloned untagged. In addition, the COPS2 and COPS5 subunits were each cloned with N-terminal FLAG®-6× His tags. To determine the tagged subunit (COPS2 or COPS5) with which the remaining untagged members should be co-expressed to generate the highest quality full complex, four 1 L cultures were grown in 3 L shake flasks and each was infected with $1 \times 10^6$ BIIC generated for each of the nine subunits (COPS1, COPS2 [tagged or untagged as specified below], COPS3, COPS4, COPS5 [tagged or untagged as specified below], COPS6, COPS7a, COPS7b, and COPS8). Two 1 L cultures were infected with FLAG-6× His-COPS2 + the remaining untagged subunits. The remaining two 1 L cultures were infected with FLAG-6× His-COPS5 + the remaining untagged subunits. The complex was purified with Anti-FLAG® M2 resin followed by size exclusion chromatography. Gel analysis demonstrates each subunit can be detected by SDS-PAGE for all samples (Fig. 3). CSN expressed and purified using the FLAG-6× His-COPS5 subunit had the more homogeneous full complex population and higher activity than that purified using the FLAG-6× His-COPS2 subunit (Figs. 3 and 4). Positive control enzyme was generated by expression of FLAG-6× His-COPS5 in HEK-293 F cells followed by pull down of endogenous CSN complex using Anti-FLAG® M2 affinity capture [7].

## 4 Notes

1. We use an adapted Sf-9 cell line that has a smaller average cell diameter and shorter doubling time than typical Sf-9 cell lines. While details pertaining to this cell line are given under Subheading 3.1, the population doubling time and average cell diameter specific to the cells you are using should be determined and procedures adjusted accordingly (*see* Chapter 1).
2. When expressing protein complexes, we tag one component and express with untagged clones of the remaining components. The entire complex is then affinity purified from the lysate through the use of the singly tagged component. Different yields, purity, and/or specific activity of the complex are obtained depending upon which component is tagged. In most cases, tagging the active component of the protein complex results in the best yields and has good activity. In some cases, tagging a different component has resulted in low yields, but higher purity and specific activity. It is best to attempt expression with several tagging strategies to obtain the best results for your specific application.

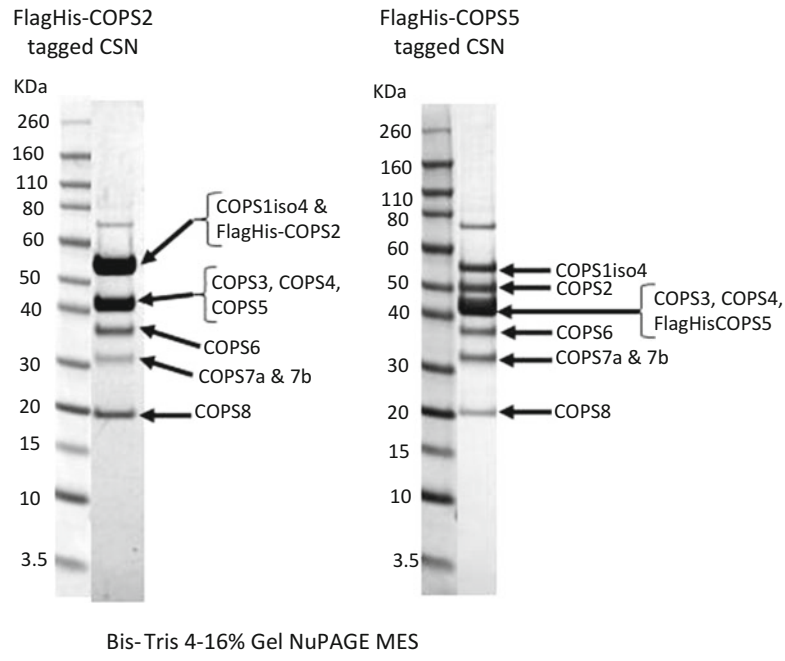


**Fig. 3** SDS-PAGE analysis of purified CSN co-expressed with either FLAG-6× His-COPS2 or FLAG-6× His-COPS5 and their respective remaining untagged complex partners. For both samples, all subunits (COPS1, COPS2, COPS3, COPS4, COPS5, COPS6, COPS7a/COPS7b, and COPS8) were detected by SDS-PAGE. CSN produced by expression with tagged COPS5 yielded a complex with more equivalent molar stoichiometry than that produced by expression with tagged COPS2

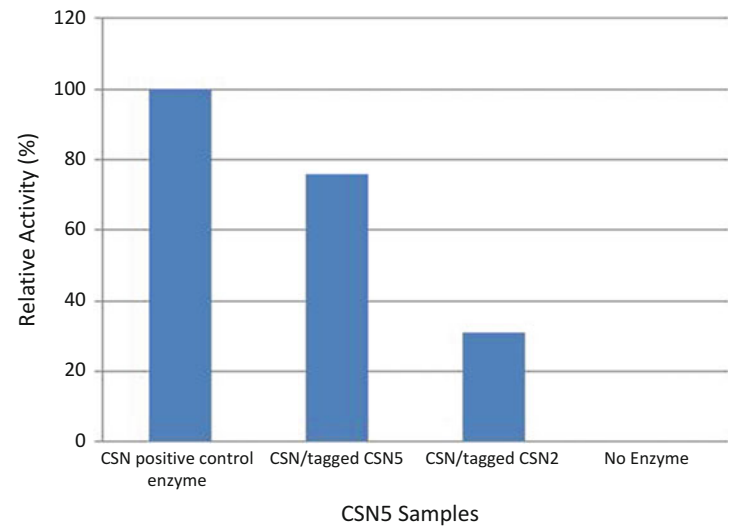


**Fig. 4** Analysis of COPS5 (CSN5) activity in the CSN eight member complex produced by co-expression of FLAG-6× His-COPS2 (tagged CSN2) or FLAG-6× His-COPS5 (tagged CSN5) and their respective remaining untagged complex partners. CSN obtained with expression using FLAG-6× His-COPS5 (tagged CSN5) has higher activity than that obtained using FLAG-6× His-COPS2 (tagged CSN2). Positive control enzyme was generated by expression of FLAG-6× His-COPS5 in HEK-293 F cells followed by pull down of endogenous CSN complex using Anti-FLAG® M2 affinity capture [7]

3. We typically use proteins with 6× His and/or FLAG® tags that are used for the mini purification of the tagged protein to determine if the remaining untagged components (i.e., the full complex) are also purified. IMAC resin and anti-FLAG® resin are used to purify proteins labeled with 6× HIS and FLAG® tags, respectively. We use a Qiagen BioSprint15 automated purification system that allows for the use of Promega MagnaHis™ or Anti-FLAG® M2 magnetic beads. However, the utilization of nonmagnetic beads for manual purification is also effective. The manufacturer's recommended buffer/elution conditions should be followed for manual purification.
4. In addition to gel analysis of the protein components, it is very helpful to have a functional activity assay available to test activity of the purified complex(es).

## Acknowledgement

This work represents a collaborative effort. I would like to thank Linda Myers for input to the BIIC generation protocol and PRC2 expression work; Yong Jiang for PRC2 purification data; Elsie Diaz for PRC2 assay data; Daniel Fornwald for CSN expression work; Ruth Lehr for CSN purification data; and Leng Nickels for CSN assay data.

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# Part V

## Recombinant Protein Production with Transformed Insect Cells

# Chapter 16

# Transforming Lepidopteran Insect Cells for Continuous Recombinant Protein Expression

**Robert L. Harrison and Donald L. Jarvis**

## Abstract

The baculovirus expression vector system (BEVS) is widely used to produce large quantities of recombinant proteins. However, the yields of extracellular and membrane-bound proteins obtained with this system are often very low, possibly due to the adverse effects of baculovirus infection on the host insect cell secretory pathway. An alternative approach to producing poorly expressed proteins is to transform lepidopteran insect cells with the gene of interest under the control of promoters that are constitutively active in uninfected cells, thereby making cell lines that continuously express recombinant protein. This chapter provides an overview of the methods and considerations for making stably transformed lepidopteran insect cells. Techniques for the insertion of genes into continuous expression vectors, transfection of cells, and the selection and isolation of stably transformed Sf-9 clones by either colony formation or end-point dilution are described in detail.

**Key words** Insect cells, Baculovirus, Baculovirus expression vector system, BEVS, Cell transformation, Genetic engineering

## 1 Introduction

### *1.1 Rationale*

Protein production with the BEVS usually involves using a recombinant baculovirus in which the polyhedrin open reading frame has been replaced with an open reading frame encoding the protein of interest [1–4]. Because the polyhedrin protein is not required for infection and replication, recombinant baculoviruses can be used to infect cultured insect cells or larvae. During infection, the polyhedrin promoter will induce the production of a very large quantity of mRNA derived from the gene of interest, which can be translated to produce large amounts of the corresponding protein. The actual yield obtained with this system can vary significantly from protein to protein, but is often measured in the 100s of mg/L of baculovirus-infected cell cultures. In addition, baculovirus-infected insect cells and larvae can perform most of the co- and post-translational protein modifications that occur in mammalian

David W. Murhammer (ed.), *Baculovirus and Insect Cell Expression Protocols*, Methods in Molecular Biology, vol. 1350, DOI 10.1007/978-1-4939-3043-2_16, 

cells, such as proteolytic processing, *N*- and *O*-glycosylation, phosphorylation, acylation, N-terminal acetylation, C-terminal methylation, and α-amidation (*see* Chapter 18). The capacity of the BEVS to produce large quantities of recombinant proteins bearing eukaryotic modifications is among its chief advantages.

A major disadvantage of the BEVS is that the yields of most membrane-bound and secreted proteins, which include many important gene products, are significantly lower (1–20 mg/L) than the yields obtained with intracellular proteins. There is some evidence to suggest that the relatively lower yields of secretory pathway proteins might reflect adverse effects of baculovirus infection on host secretory pathway function [5]. This observation led to the development of a new approach to recombinant protein production, which involved stable transformation of lepidopteran insect cell lines with plasmids encoding the protein of interest under the control of a constitutively active promoter [6]. The promoter initially used to develop this approach was derived from a baculovirus immediate early gene, *ie-1* [7, 8]. Plasmids encoding foreign proteins under *ie-1* control can integrate into the cellular genome and, because the *ie-1* promoter is active in uninfected lepidopteran insect cells, this approach circumvents the need to infect the cells with a recombinant baculovirus.

The first study utilizing this approach showed that transformed Sf-9 cell lines constitutively expressing *E. coli* β-galactosidase under the control of the *ie-1* promoter produced far less β-galactosidase than Sf-9 cells infected with a conventional baculovirus expression vector. This was expected, as the *ie-1* promoter is significantly weaker than the polyhedrin (*polh*) promoter and β-galactosidase, a cytoplasmic protein, can be produced at very high levels by the conventional baculovirus-insect cell system. However, this study also showed that transformed Sf-9 cells constitutively expressing human tissue plasminogen activator (t-PA), a secretory pathway protein, produced about as much extracellular product under *ie-1* control as the Sf-9 cells infected with a conventional baculovirus vector [6]. While the baculovirus-infected cells produced more total (intracellular and extracellular) t-PA, most of the t-PA produced by this conventional system was not secreted. Hence, the efficiency of t-PA secretion was considerably higher with the uninfected, transformed cell lines than with the baculovirus-infected cells, supporting the idea that baculovirus infection might, indeed, have an adverse effect on host secretory pathway function.

The aforementioned study was the first to demonstrate that stably transformed lepidopteran insect cell lines could be used to constitutively produce a foreign secretory pathway protein at a level comparable to that obtained with the conventional BEVS. Since then, Sf-9 cells have been transformed to constitutively express many other foreign genes under *ie-1* control [9]. The results of these subsequent studies confirmed the trends originally observed

by Jarvis and coworkers and revealed other advantages of using uninfected, transformed lepidopteran insect cell lines to express certain types of gene products (*see* **Note 1**). In addition, the original work has been reproduced and extended by the development of new expression constructs [10–20], the use of other selection markers [11, 12, 21], and the use of other lepidopteran insect cell lines [12, 14, 15, 22]. High-density culture systems for larger-scale expression of recombinant protein from stably transformed lepidopteran insect cell lines also have been evaluated [23, 24].

### 1.2 Basic Approach

The first step in producing a stably transformed lepidopteran insect cell line designed to continuously express a foreign protein is to insert a DNA sequence encoding the foreign protein of interest into a plasmid vector containing a promoter that is active in the parental cell line. The *ie-1* and *ie-2* promoters, the latter derived from another baculovirus immediate early gene [25], have been widely used for this purpose [6, 10, 11, 13], as have host promoters derived from a *B. mori* (silkworm) actin gene [14] and a *Drosophila melanogaster* (fruit fly) heat shock protein gene (*hsp70*) [12]. Baculovirus-derived enhancer elements, known as homologous DNA regions (*hr*s), have been used to stimulate *ie-1*-mediated transcription in stably transformed insect cells [10]. Interestingly, it has been shown that a baculovirus-derived *hr* element also can stimulate *B. mori* actin promoter-mediated transcription in transiently transfected insect cells, suggesting that this element might be able to augment actin-mediated expression in transformed cells, as well [26]. While this chapter focuses on the use of vectors for constitutive expression, vectors that provide for inducible expression in lepidopteran insect cells have also been developed and tested [16, 18–20, 27].

Plasmids encoding the gene of interest under the control of an appropriate promoter are introduced into lepidopteran insect cells by conventional DNA transfection procedures. In addition, one must introduce a constitutively expressible antibiotic resistance marker to enable selection of transformed cells that have incorporated functional copies of the gene(s) of interest into their genomic DNA. The antibiotic resistance marker may be placed either on the same plasmid as the gene of interest [13] or on a separate plasmid [6]. As for genes encoding proteins of interest, the antibiotic resistance markers are typically expressed under the control of baculovirus immediate early or insect cell promoters. A wide variety of resistance markers have been used, including neomycin (G418) [6], hygromycin B [21], puromycin [12], Zeocin™ [11], and blasticidin S [28]. However, it is important to recognize that lepidopteran insect cells maintained in the presence of the antibiotics routinely used for cell culture can be resistant to some of the antibiotics listed above [29].

Transformation vectors of the sort described in this chapter integrate into the chromosomal DNA of insect cells by illegitimate recombination. However, we should note that lepidopteran insect cells also have been transformed with *piggyBac* transposon-based vectors [15, 17, 27, 30, 31]. In the latter type of vector, genes encoding the recombinant protein of interest and a selectable marker are cloned between the inverted terminal repeats of a *piggyBac* transposon. This construct is then transfected into insect cells together with a helper plasmid encoding the *piggyBac* transposase. The transposase expressed by the helper plasmid catalyzes the transposition and precise integration of the *piggyBac* transposon carrying the gene of interest and marker gene into a tetranucleotide (TTAA) target site in host cell DNA. Cells transformed by these *piggyBac*-based vectors can be selected for antibiotic resistance using the same procedures as are used with standard transformation vectors [17, 27, 31]. An alternative (or supplement) to selection for drug resistance involves identifying and sorting transformed cells by fluorescence. If the vector used for cell transformation carries an expression cassette for a fluorescent protein such as green fluorescent protein (GFP), or if the coding sequence of the gene of interest is fused to a fluorescent protein coding sequence, then one can use fluorescence-activated cell sorting (FACS) with a flow cytometer to enrich for transformed cell subpopulations [15, 32].

Once selected, individual cells can be isolated and expanded to obtain transformed clones. Two methods that we have used to obtain clonal transformed insect cell derivatives are detailed below and outlined in Fig. 1. It is important to isolate multiple transformed clones and compare their abilities to produce the protein of interest, because antibiotic resistance does not guarantee expression of the unselected marker(s) and individual clones expressing the gene of interest typically exhibit a wide range of expression levels [6, 14]. Finally, once transformed clones expressing the gene of interest have been identified, it is important to recognize that this is not guaranteed to be a stable genetic trait. In order to validate the claim that one has isolated a "stably" transformed clone, it is necessary to monitor expression of the unselected marker routinely over a large number of passages. In our experience, transformed lepidopteran insect cells can express newly acquired transgenes over hundreds of passages. Furthermore, while the expression level is not necessarily constant from passage to passage, it should not steadily decline with increasing passage number if the transformed clone is a genetically stable cell line. In a recent study, we directly examined the impact of the presumed metabolic load imposed by the expression of multiple transgenes and found that the expression of six separate transgenes had no major impact on the growth properties or phenotypic stabilities of a transformed Sf-9 cell line, even after 300 passages (1.5 years) in continuous culture [27].

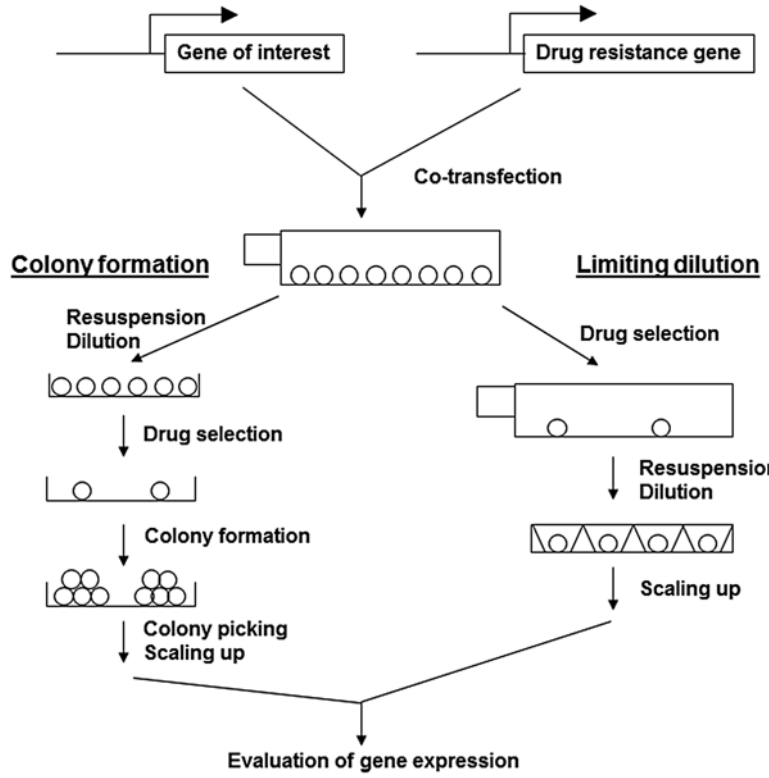


**Fig. 1** An overview of two methods for generating monoclonal isolates of transformed lepidopteran insect cells. Cells are co-transfected with IE-1 expression plasmids encoding the gene(s) of interest and a selectable drug resistance marker. After drug selection, clonally derived cell lines can be isolated either by picking and expanding colonies formed on petri dishes or by growing lines from individual drug-resistant cells deposited into the wells of 96-well plates

## 2 Materials

1. Cell lines: Sf-9 (ATCC: #CRL-1711; *see* **Note 2**) can be obtained from the American Type Culture Collection (Manassas, VA 20108; www.atcc.org) or from Life Technologies (Carlsbad, CA; www.lifetechnologies.com).
2. Cell media: Grace's Insect Medium, Hink's TNM-FH medium, and Pluronic® F-68 are available from Sigma-Aldrich Corporation (St. Louis, MO; www.sigma-aldrich.com; *see* **Note 3**).
3. Plastics: Falcon and Corning flasks, petri dishes, and multiwell plates for cell culture are available from Fisher Scientific (Pittsburgh, PA; www.fishersci.com).
4. Hemacytometer and cell counters: available form Fisher Scientific.

5. Fetal bovine serum is available from Life Technologies (*see* **Note 4**).
6. Conventional cell culture antibiotics: Amphotericin B (Fungizone®) and gentamicin can be obtained from Life Technologies.
7. Antibiotics for selection of transformed cells: G418 (Geneticin®), hygromycin B, Zeocin™, and blasticidin S are available from Life Technologies.
8. Enzymes for constructing expression plasmids: Restriction endonucleases, calf intestinal alkaline phosphatase, DNA polymerase I (Keno fragment), and T4 DNA polymerase can be obtained from New England Biolabs (Ipswich, MA; www.neb.com).
9. Plasmids: The pIEHR series of expression vectors (Fig. 2), pDIE1HR1 (Fig. 1, Chapter 18), pDIE1-(fluorescent protein)-TOPO.4 series of expression vectors (Fig. 1, Chapter 18), and the selection plasmids pIE1Neo and

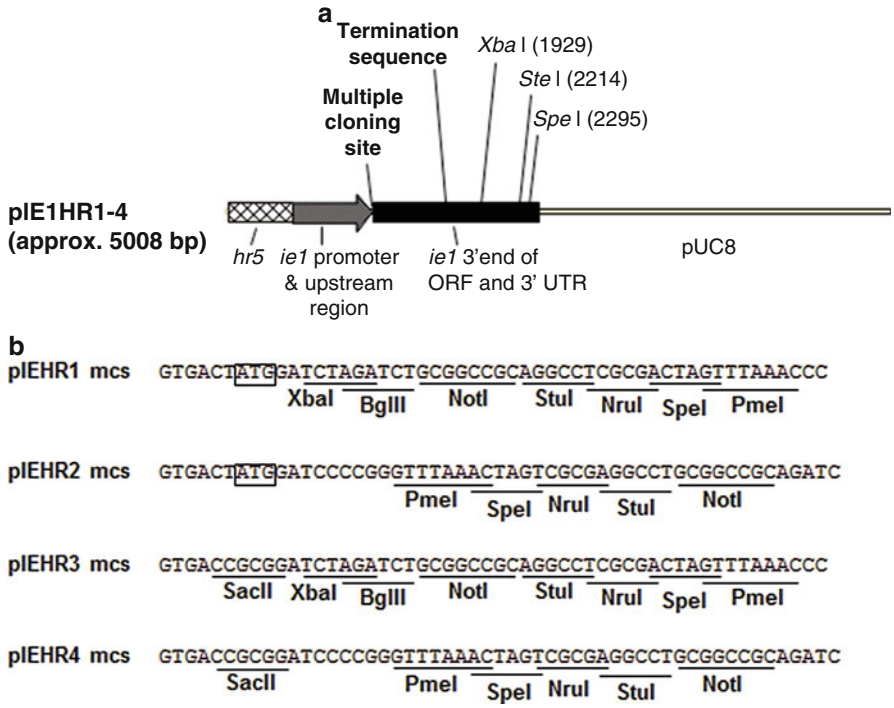


**Fig. 2** (**a**) Features of the pIE1HR series of plasmids used for transformation and constitutive gene expression in lepidopteran insect cells. The *hr5* enhancer, *ie-1* promoter and termination sequence, and multiple cloning site are indicated. Restriction sites that are also present in the multiple cloning sites are noted. (**b**) Sequences of the multiple cloning sites for the pIE1HR series (1–4) with restriction sites *underlined*. pIE1HR1 and pIEHR2 utilize the native *ie-1* start codon (*boxed* ATG) for translation initiation. In pIE1HR3 and pIE1HR4, the *ie-1* start codon has been replaced with a *Sac* II site

pIE1Hygro are available from Dr. Donald Jarvis upon request. A series of vectors that use the same *hr5-ie-1* enhancer-promoter arrangement (the pIEx™ series) is available for purchase from EMD Millipore (Billerica, MA; www.emdmillipore.com). In addition, Life Technologies sells expression plasmids (the pIB and pIZ series) that carry drug resistance genes for blasticidin S and Zeocin™.

10. Chemicals: Buffers (HEPES, Tris), NaCl, $CaCl_2$, ethylenediaminetetraacetic acid (EDTA), 100 % ethanol, ethidium bromide, glucose, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), sodium acetate, potassium acetate, potassium hydroxide, formic acid, glacial acetic acid, Tris-equilibrated phenol, and chloroform can be obtained from Fisher Scientific. Molecular biology-grade reagents should be purchased when available.
11. Deionized, distilled, DNAse- and RNAse-free water: If a water purification system and an autoclave are not available, bottles of sterile water suitable for molecular biology research can be purchased from Fisher Scientific.
12. Benchtop pH meter: Several brands are available from Fisher Scientific.
13. Solutions for vector preparation, miniprep and maxiprep DNA isolation and purification, and cell transfection: 1:1 phenol–chloroform, with phenol pre-equilibrated to pH 8.0 with Tris buffer; 3 M sodium acetate, brought to pH 5.2 with glacial acetic acid; Solution I (50 mM glucose, 10 mM EDTA, and 25 mM of a Tris–HCl, pH 8.0, which is prepared from a Tris buffer stock solution brought to pH 8.0 with HCl); Solution II (1.0 % sodium dodecyl sulfate, 0.2 N NaOH); Solution III (3 M potassium acetate, 1.8 M formic acid); TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA); Transfection buffer (140 mM NaCl, 125 mM $CaCl_2$, and 25 mM HEPES, pH 7.1, prepared from a HEPES stock brought to pH 7.1 with potassium hydroxide).
14. Liposome transfection reagents: Cellfectin® II Reagent can be obtained from Life Technologies.
15. Cloning reagents (bacteria): Competent *E. coli* are available from Promega (Madison, WI; www.promega.com).
16. Cloning reagents (bacteria media, etc.): LB broth, 2×YT broth, LB agar, ampicillin, and 100 mm plastic petri dishes for agar plates are available from Fisher Scientific.
17. Additional equipment for vector preparation: Vortex mixers, microcentrifuges, standard laboratory incubators and incubating shakers capable of maintaining 37 °C, and UV-Vis spectrophotometers are available from Fisher Scientific. Floor model centrifuges, ultracentrifuges, rotors, and other centrifuge

accessories are available from Thermo Scientific (Waltham, MA; www.thermoscientific.com) or Beckman Coulter (Brea, CA; www.beckmancoulter.com)

18. Micropipetters can be obtained from Fisher Scientific or Mettler Toledo (Greifensee, Switzerland; us.mt.com). Pasteur pipettes and vacuum grease are available from Sigma-Aldrich Corporation. Glass cloning cylinders can be obtained from Bellco Glass, Inc. (Vineland, NJ; www.bellcoglass.com).

## 3 Methods

### 3.1 Insertion of the Gene of Interest into an Expression Plasmid

Prior to cell transformation, the gene of interest must be placed into a plasmid under the control of a promoter that is constitutively active in uninfected cells. We routinely use a set of plasmids, designated "immediate early expression plasmids" in which the gene of interest is inserted between the AcMNPV *ie-1* promoter/upstream region and the *ie-1* transcriptional termination signal. The *ie-1* promoter is linked in *cis* to the AcMNPV *hr5* enhancer (Fig. 2) [10]. This enhancer stimulates *ie-1*-mediated transcription in transfected or transformed insect cells when these cells are infected with a baculovirus or co-transfected with a plasmid encoding the baculovirus IE1 protein [7, 33]. These four immediate early expression plasmids differ in the arrangement and selection of restriction sites available for inserting the gene of interest and also by the presence or absence of the *ie-1* start codon. pIE1HR1 and pIE1HR2 both include the native *ie-1* start codon upstream of the multiple cloning site. These plasmids were originally designed to express genes of interest without their own ATGs, after being inserted in-frame with the upstream, native translational initiation site. Conversely, the *ie-1* ATG was replaced with a *SacII* site in pIE1HR3 and pIE1HR4 site. Hence, these vectors were designed to express genes of interest with their own ATGs, which can be inserted without considering the open reading frame. We have also constructed plasmids capable of expressing two genes at once. These "dual" immediate early expression plasmids consist of a single copy of *hr5* flanked by two copies of the *ie-1* promoter oriented in opposite directions [16, 34, 35] (also *see* Fig. 1 and Chapter 18). This expression plasmid can be used to produce a cell line that expresses two genes of interest for various applications, including simultaneous production of both partners of a heterodimer.

None of the expression plasmids described above have selectable markers. Thus, to isolate transformed insect cells, we routinely co-transfect with a separate selection plasmid that encodes a neomycin-(pIE1Neo) [6] or hygromycin B-(pIE1Hygro) [21] resistance marker. The following is an outline of the steps we use to insert a foreign gene into one of the expression vectors detailed in

Fig. 2, and to produce a preparation of the resulting plasmid that can be used in transfecting insect cells.

1. Digest one of the pIEHR series of vectors with restriction enzymes that cut at the sites needed to insert the gene.
2. Dephosphorylate the ends with calf intestinal alkaline phosphatase. If the restriction fragment carrying the gene of interest has blunt termini, then blunt the ends of the vector with the Klenow fragment of *E. coli* DNA polymerase I (for recessed 3′ ends) or T4 DNA polymerase (for the protruding 3′ end produced by cleavage with *Sac* II).
3. Bring the vector preparation volume to 500 μL with distilled water. Add 500 μL of a 1:1 mixture of phenol (equilibrated to pH 8.0) and chloroform. Vortex briefly and separate the organic and aqueous phases by microcentrifugation (12,500 × *g* for 5 min). Transfer the aqueous (top) phase to a fresh tube and add 50 μL 3 M sodium acetate (pH 5.2) and 1 mL 100 % ethanol. Vortex briefly and pellet precipitated DNA by microcentrifugation (14,000 × *g* for 15 min).
4. Prepare the gene of interest as a restriction fragment. Run aliquots of the vector and the restriction fragment carrying the gene of interest on an agarose gel to evaluate the relative quantities of each.
5. Set up a ligation reaction with the gene of interest present at a 2:1 or greater molar excess over the vector.
6. Transform competent *E. coli* cells with the ligation products. Spread transformed cells on LB-agar plates containing 50 μg/mL ampicillin and incubate plates overnight at 37 °C to allow for colony growth.
7. Set up test tubes containing 2.5 mL of 2× YT medium with 50 μg/mL ampicillin. Inoculate each tube with a colony. Shake tubes at 225 rpm 8 h to overnight at 37 °C.
8. Pipette 1.5 mL of each culture into individual microfuge tubes. Pellet bacteria by brief microcentrifugation (14,000 × *g* for 1 min). Pour off supernatant, drain the pellets, and place tubes on ice.
9. Isolate plasmid DNA from the pellets by the alkaline lysis method [36]. To start, add 100 μL Solution I. Vortex to resuspend the pellets.
10. Add 200 μL Solution II to each tube. Mix by inversion. The contents of the tubes should be viscous.
11. Add 150 μL Solution III and mix by inversion. A white flocculent precipitate should appear.
12. Pellet the precipitate by microcentrifugation (14,000 × *g* for 5 min). Transfer the supernatant to a fresh Eppendorf tube.

Extract the plasmid DNA with phenol–chloroform and precipitate with ethanol as described in **step 3**.

13. Resuspend DNA pellets in 50–100 µL deionized, distilled $H_2O$. Screen the plasmids for the presence and proper orientation of the inserted gene by restriction mapping.
14. Use 0.5 mL of leftover miniprep culture from an appropriate clone to inoculate 200 mL of 2× YT containing 50 mg/mL ampicillin in a 500 mL flask. Incubate with shaking (225 rpm) overnight at 37 °C.
15. Pellet bacteria by centrifugation (5000 × *g* for 10 min). Decant supernatant and resuspend pellets in a total of 4 mL Solution I.
16. Add 8 mL Solution II and incubate on ice with gentle shaking for 5 min. Add 6 mL Solution III and shake again on ice for 5 min.
17. Pellet flocculent material by centrifugation (10,000 × *g* for 15 min).
18. Transfer supernatant to a 50 mL conical tube. Fill tube with 100 % ethanol, mix by inversion, and pellet nucleic acid by centrifugation (5000 × *g* for 15 min). Decant supernatant and drain pellet.
19. To form CsCl-ethidium bromide equilibrium density gradients, resuspend the pellet in a volume of TE appropriate for the type of tube to be used for ultracentrifugation. Add 1.01 g CsCl per g of suspended pellet and 100 µL 10 mg/mL ethidium bromide per 5 g of suspended pellet. Vortex to dissolve the CsCl. Transfer suspension to a tube suitable for centrifugation in an ultracentrifuge rotor.
20. Centrifuge the tubes at 20 °C at a relative centrifugal force appropriate to the rotor [37]. For example, if using a Beckman Type 70.1 Ti rotor, the tubes should be centrifuged at 331,000 × *g* for 24 h.
21. After centrifugation, collect the band of closed circular plasmid DNA from the gradients and transfer to a 15 mL conical tube. To remove ethidium bromide from the plasmid DNA, add an equal volume of water-saturated butanol and vortex. Allow the aqueous and butanol phases to separate. Remove the top (butanol) phase and discard. Repeat the extraction until the top phase is clear.
22. Transfer DNA to a 50 mL conical tube, dilute fivefold with deionized, distilled $H_2O$, and precipitate the DNA with 100 % ethanol. Pellet DNA and resuspend in TE pH 8.0.
23. Quantitate the DNA by measuring absorbance of a dilution in TE at 260 nm and verify its structure by restriction endonuclease mapping.

### 3.2 Transfection

As outlined in Fig. 1, cells are co-transfected with a mixture of an expression plasmid bearing the gene of interest and another carrying a drug resistance gene. The following protocol describes the steps for co-transfection of Sf-9 cells using the calcium phosphate precipitation method [38] prior to antibiotic selection. An alternative method is transfection with DNA packaged into liposomes formed by a commercial cationic lipid reagent (such as Cellfectin® II from Life Technologies), which should be done following the vendor's recommendations. Both methods result in an efficiency of transfection sufficient for producing stably transformed cells, but it has been reported that liposome-mediated transfection results in a lower number of copies of the expression vector integrated into the cells' chromosomes [39].

1. Seed each of two 25 $cm^2$ T-flasks with $2 \times 10^6$ Sf-9 cells in a final volume of 5 mL of antibiotic-free medium. One of the flasks will be transfected in the presence and the other in the absence of the selectable marker to verify the efficacy of the selection process. The cells must be healthy (97–98 % viability) and grown in the absence of antibiotics.
2. Allow cells to attach for 1 h.
3. Prepare the plasmid DNAs for transfection as follows:
   (a) Place 1 μg of the selectable marker and 2–20 μg of the immediate early expression plasmid(s) encoding the protein(s) of interest into a single microfuge tube (*see* **Note 5**). Place the same amount of the immediate early expression plasmid(s) encoding the protein(s) of interest, with no selectable marker, into a second microfuge tube.
   (b) Ethanol-precipitate to sterilize the DNAs and prevent bacterial contamination of the transfections. Use 70 % ethanol to surface-sterilize the microfuge tubes, as well.
   (c) Aseptically transfer 0.75 mL of transfection buffer to the tubes containing the DNA pellets and gently resuspend the DNAs in this buffer.
4. Remove the TNM-FH medium from both cell cultures. Rinse twice with 5 mL of complete Grace's medium (Grace's medium supplemented with 10 % fetal bovine serum, 1.25 μg/mL amphotericin B, and 25 μg/mL gentamycin). Remove the last rinse from the flasks completely and add 0.75 mL of complete Grace's medium to each flask.
5. Add the DNA mixtures, dissolved in transfection buffer (*see* **Note 6**), to the appropriate flasks. Manually rock the flasks from side to side briefly and gently to mix the transfection buffer and Grace's medium.
6. Incubate the flasks at 28 °C for 2 h. The transfection mixture should take on a milky white appearance. If this does not happen, then check the pH of the transfection buffer.

7. Drain the transfection mixtures from the cells. Rinse twice with complete TNM-FH medium (TNM-FH supplemented with 10 % fetal bovine serum and antibiotics).
8. Feed the cells with 5 mL complete TNM-FH and incubate overnight at 28 °C.

### 3.3 Selection and Isolation of Transformed Clones by the Colony Formation Method

This procedure takes advantage of the propensity of insect cells to form discrete colonies when cultured at extremely low densities. Selection and amplification of transformed cell clones requires long-term culture (approximately 1 month). It is therefore critical to be extremely meticulous to avoid microbial contamination.

1. Dislodge cells from the surface of the flasks obtained in Subheading 3.2, **step 8**. We routinely use the medium in the flask and a Pasteur pipette to squirt the cells off the plastic surface ("fire-hose" method). An alternative method for dislodging cells is given in Chapter 9.
2. Dilute each culture to a total volume of 30 mL with complete TNM-FH.
3. Set up 60-mm petri dishes with 3 mL complete TNM-FH. Plate diluted cells using the split ratios given below, depending upon the amount of expression plasmid used for transfection (*see* **Note 7**):
   (a) For 2 μg of DNA, seed at 1:60 and 1:75
   (b) For 5–10 μg of DNA, seed at 1:45 and 1:60
   (c) For 20 μg of DNA, seed at 1:30 and 1:45
4. Seal the dishes in a humidified plastic baggie to minimize evaporation of the medium. Incubate the dishes overnight at 28 °C.
5. Replace the medium with complete TNM-FH containing the selection antibiotic (4 mL per 60-mm dish; *see* **Note 8**).
6. Incubate the dishes at 28 °C for 1 week. After 3–4 days, all the control cells transfected with the expression plasmid alone should be dead (*see* **Note 9**). If the control cells are still alive after 1 week of antibiotic treatment, then the selection procedure has failed.
7. Remove the old medium, wash gently with complete TNM-FH, and add fresh complete TNM-FH plus the selection antibiotic. Incubate for another week at 28 °C.
8. Remove the old medium, wash cells gently with complete TNM-FH, and add fresh complete TNM-FH without any antibiotic. Incubate dishes at 28 °C until large colonies of densely packed cells form. The colonies should be at least 2 mm in diameter. Smaller, relatively less dense colonies are unlikely to survive the cloning procedure (*see* **Note 10**). Larger colonies are likely to be lost by detaching from the surface of the plate.

9. Drain one 60-mm dish to pick the colonies. Do not drain more than one dish at a time, as the cells will dry out in the additional dishes and die before you can pick the colonies.
10. Using forceps, dip a cloning cylinder in alcohol and flame-sterilize it. Dip one end of the sterilized cylinder into a dollop of vacuum grease on a petri dish. Place the cylinder, greased-end down, onto the drained dish around a well-isolated colony. The vacuum grease must form a seal between the cylinder and the dish.
11. Add 100 μL of complete TNM-FH to the interior of the cloning cylinder and use a micropipettor to gently disperse the cells in the colony. Transfer the dispersed cells to a single well in a 96-well plate.
12. Monitor the cells daily. The cells may grow to 80–90 % confluency in 3–7 days. Approximately half the colonies will fail to grow. Amplify each surviving colony in stepwise fashion by transferring the cells first to a 24-well plate, then to a 6-well plate, and then to a 25-cm$^2$ flask. Allow the cells to grow close to confluency at each step.
13. When the 25-cm$^2$ flask culture is nearly confluent, split it 1:3. Use two of the resulting 25-cm$^2$ flasks, which we define as passage 1, to prepare P1 freezer stocks (*see* **Note 11**). Use the remaining P1 culture for screening and further amplification.

### *3.4 Selection and Isolation of Transformed Clones by the Limiting Dilution Method*

This procedure is a more reliable way to obtain verified, single cell clones than the colony-formation method. However, it is unclear whether all established insect cell lines are amenable to single cell cloning by the limiting dilution method.

1. Drain media from the flasks from **step 8** of the co-transfection procedure (Subheading 3.2) and add 5 mL complete TNM-FH plus selection antibiotic.
2. Incubate flasks 1 week at 28 °C. All the cells in the control flasks should be dead. Many cells in the flasks co-transfected with the expression plasmid and the resistance marker will also die, but there should be a significant number of surviving cells.
3. Use the fire-hose method to detach cells from the flasks and perform a cell count.
4. Dilute the cells to a concentration of about 5 cells/mL with Sf-9 cell-conditioned medium (medium from Sf-9 exponential growth phase culture; *see* Chapter 1).
5. Dispense 200 μL of the diluted cells into multiple wells of a 96-well plate. Check with a phase-contrast microscope to determine if most wells contain a single cell. If this is not the case, then adjust your dilution as necessary and try again. Once you find the appropriate dilution, dispense enough cells to fill

an entire 96-well plate because about half of the single cell clones will probably fail to grow.

6. Monitor the wells daily for cell growth. As the clones grow, amplify each one stepwise as described in **step 12** under Subheading 3.3.
7. Process the 25-cm$^2$ flask, P1 cultures as described in **step 13** of the colony formation method (Subheading 3.3).

### 3.5 Screening for the Presence and Expression of the Gene of Interest

Once multiple antibiotic-resistant insect cell clones have been isolated, they must be screened to determine if they contain and express the gene of interest. Southern blotting of restriction enzyme-digested genomic DNA from the transformed cells will reveal the presence of the gene of interest integrated into cellular DNA [5]. A simple dot-blot hybridization of cytoplasmic RNA from the transformed cells [40] will reveal whether or not the gene is transcribed, while Northern blotting [41], S1 nuclease [42], primer extension [43], RNAse protection assays [44], and/or rapid amplification of cDNA ends (RACE) [45] can be used to examine transcription of the gene of interest in more detail. If an antibody is available against the protein of interest or an epitope tag fused to the protein of interest [46], then many standard techniques, such as indirect immunofluorescence [47], immunoprecipitation [5], and Western blotting [48], can be used to detect the gene product in transformed cells. These techniques also can provide some information about the localization of the protein in transformed cells. Obviously, none of these detection methods guarantee that the transformed cells are producing an active, biologically functional protein of interest. Hence, depending on the application, it can be critically important to perform a functional assay of transformed insect cell clones containing and expressing the gene of interest. Finally, as mentioned above, it should be recognized that transformed insect cell clones are not guaranteed to be genetically stable, so it is important to monitor chosen clones for retention of the gene of interest as those cells are maintained through increasingly higher passages in the lab.

## 4 Notes

1. A number of studies indicate that stably transformed insect cells can produce higher quality recombinant proteins and circumvent some difficulties associated with the conventional baculovirus-infected cell approach, particularly for functional analyses of secretory pathway proteins. Baculoviruses produce a cysteine protease during infection that can degrade foreign proteins and further reduce the yield of secreted protein [49, 50]. A comparative study of β1,3-N-acetylglucosaminyltransferase

2 (β3GnT2) expression by Tn-5 cells infected with a baculovirus vector or stably transformed with the β3GnT2 gene [51] revealed that the infected Tn-5 cells produced extensively degraded β3GnT2, whereas the stably transformed Tn-5 cells produced intact β3GnT2. (Note that Tn-5 is the *Trichoplusia ni* BTI Tn-5B1-4 cell line [52] commercially available from Life Technologies as High Five™ cells). Furthermore, the specific activity of β3GnT2 purified from the stably transformed Tn-5 cells was 1.6- to 2.9-fold higher than that purified from the baculovirus vector-infected cells. Another study showed that patch clamp analysis of Sf-9 cells expressing the bovine $GABA_A$ receptor was significantly easier to perform on cells stably transformed with the $GABA_A$ receptor gene than on cells infected with a baculovirus vector encoding the $GABA_A$ receptor [53]. Finally, the human β2-$_a$drenergic receptor expressed in stably transformed Sf-9 cells was able to function in concert with the endogenous insect cell adenylyl cyclase system, which was not observed when the β2-adrenergic receptor was expressed using a standard baculovirus vector [54].

While the above studies demonstrate that the stably transformed cell approach often presents advantages over the infected-cell approach, we also should note that this is not always the case. In a study comparing expression of human estrogen receptor beta (ERβ) in stably transformed IPLB-Sf21-AE (Sf-21) [55] cells versus baculovirus-infected Sf-21 cells, the yield of active ERβ from the infected cells was tenfold higher than that obtained with stably transformed cells expressing ERβ under *ie-2* control [56]. The stably transformed ERβ-expressing Sf-21 cell line exhibited reduced cell growth and viability relative to untransformed Sf-21 cells, suggesting a possible explanation for the superior expression of active ERβ with a baculovirus vector in this instance.

2. For transformations, we usually start with the Sf-9 subclone of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE [55]. We maintain Sf-9 cells in suspension culture in TNM-FH medium supplemented with 10 % (v/v) fetal bovine serum and 0.1 % (w/v) Pluronic® F-68 [57]. For cell transformation, we only use Sf-9 cells that have never been exposed to antibiotics. This practice follows from the observation that Sf-9 cells maintained in 1.25 μg/mL amphotericin B and 25 μg/mL gentamycin were highly resistant to neomycin selection [28]. We also have successfully transformed Tn-5 cells [52] using the procedures described herein, though we were unable to isolate single cell clones by limiting dilution in that particular study [34].
3. Grace's and TNM-FH media also can be prepared according to published recipes [38]. All media and media components should be stored in the dark at 4 °C.

4. Although heat inactivation of serum to be added to insect cell medium has been a standard practice, we no longer heat-inactivate the serum used in our growth media because studies performed at Thermo Scientific HyClone have shown that heat inactivation has no positive influence on insect cell culture (*see* "Heat Inactivation—Are You Wasting Your Time?" in *Art to Science in Tissue Culture*, Vol. 15, No. 1). Different batches of serum differ in their capacity to support insect cell growth so it is important to test new serum lots before making a large-scale purchase.
5. Within the range of 2–20 μg of expression plasmid, there does not appear to be any significant difference in the transformation efficiencies or expression levels obtained with transformed clones produced by G418 selection. In fact, there are typically fewer total G418-resistant colonies obtained with larger amounts of expression plasmid. This is why lower split ratios are used to isolate clones in **step 3** under Subheading 3.3 after transfection with higher quantities of expression plasmid.
6. The pH of the transfection buffer can drastically affect the efficiency of transfection and must be adjusted precisely. After preparation, the transfection buffer must be filter-sterilized and stored at 4 °C.
7. Because the cells in the flask are suspended in a total of 30 mL medium, seeding 1 mL of this suspension into a dish yields a 1:30 split. Higher split ratios are achieved by seeding less than 1 mL or performing further dilutions with complete TNM-FH. To ensure that well-isolated colonies are obtained, a range of split ratios should be used (1:20–1:100).
8. We have routinely used G418 and hygromycin B at a concentration of 1.0 mg/mL and also have used Zeocin™ at sequentially increasing concentrations from 0.3 to 1.0 mg/mL. After addition of the antibiotic, the medium is filter-sterilized and supplemented with 10 % fetal bovine serum. Conventional cell culture antibiotics (1.25 μg/mL amphotericin B and 25 μg/mL gentamycin) also can be added at this time to prevent contamination.
9. If the control cells survive antibiotic treatment, then (a) determine if the cells were previously exposed to antibiotics and (b) check the expiration date of the antibiotic stock used for selection.
10. Hints for successfully picking and amplifying transformed colonies:
    (a) Small, less dense colonies will not grow. However, colonies that are too large will detach from the surface of the dish and be lost when the growth medium is removed.

(b) Vacuum grease does not seem to create a significant contamination problem. However, one can surface-sterilize the grease by placing a dollop on a petri dish under UV light in a laminar flow hood.

(c) To avoid having the colonies on a plate dry out after removal of growth medium, place cloning cylinders over no more than four colonies before adding medium to the interior of each cylinder.

(d) To obtain single cell suspensions, triturate individual colonies with a micropipettor. If the cells are still clumpy after being seeded into a 96-well plate, then they can be dispersed by additional trituration. Examine the plates with a phase-contrast microscope after picking colonies to make sure that most of the cells from each colony have been collected. If not, then add more medium and use a micropipettor to recover the additional cells.

(e) We typically pick 12 antibiotic-resistant colonies of cells transformed with a single gene of interest. Some colonies will stop growing, grow slowly, or become contaminated during the amplification process. However, most of the survivors typically contain and express the unselected marker. It is typically necessary to start with larger numbers of antibiotic-resistant colonies (or single cell clones) to isolate transformed clones containing and expressing multiple unselected markers.

11. Freezer stocks are prepared by gently removing cells from the 25-$cm^2$ flask surface, pelleting the cells by low-speed centrifugation (approximately $120 \times g$) for 1 min, and resuspending in complete TNM-FH plus 10 % DMSO. Optimal cell cryopreservation and recovery is achieved with a cooling rate of −1 °C/min. The Cryo 1 °C Freezing Container ("Mr. Frosty") from Nalgene (Rochester, NY; catalog #5100-0001) provides this cooling rate. Subsequently, the cells should be placed in liquid nitrogen for long-term storage.

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# Chapter 17

# Stable Drosophila Cell Lines: An Alternative Approach to Exogenous Protein Expression

**Marija Backovic and Thomas Krey**

## Abstract

Recombinant protein production has become an indispensable tool for various research directions and biotechnological applications in the past decades. Among the numerous reported expression systems, insect cells provide the possibility to produce complex target proteins that require posttranslational modifications. Stable expression in Drosophila S2 cells represents an attractive alternative to the widely used baculovirus expression system, offering important advantages in particular for difficult-to-express proteins, e.g., membrane proteins or heavily glycosylated multi-domain proteins that are stabilized by a complex disulfide pattern. Here we present the methodology that is required for the generation of stable Drosophila S2 cell transfectants and for production of recombinant proteins using those transfectants.

**Key words** Protein expression, S2, Drosophila, Stable cell line, Transfection, Membrane protein, Glycoprotein

## 1 Introduction

Recombinant protein expression is an essential prerequisite for structural and functional analysis of proteins derived from a variety of organisms. The growing complexity of these proteins requires an expression host that is capable of ensuring a native fold as well as posttranslational modifications that are essential for protein function and/or structure. The use of insect cells is constantly increasing because culturing of these cells is relatively cheap, and they are capable of producing mg amounts of accurately translated and correctly processed heterologous proteins. Two main complementary expression systems are available for expression in insect cells: the baculovirus expression vector system (BEVS) and stable *Drosophila melanogaster* Schneider 2 (S2) cell lines.

### 1.1 Limitations of the BEVS

Baculovirus-based expression of recombinant proteins is an easy and fast method of producing large amounts of correctly folded proteins and has therefore been widely used in the past decades.

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However, the most commonly used baculovirus systems use strong promoters (such as the polyhedrin promoter) in order to drive high-level protein expression. Because polyhedrin is expressed in the late-phase of the baculovirus cycle, protein expression coincides with baculovirus-induced cell lysis (~72 h post-infection). This leads to increased protease levels in the medium, thereby decreasing the yield of recombinant protein [1].

Proteins that are targeted to the secretory pathway require assistance of cellular folding factors present in the lumen of the endoplasmic reticulum (ER). The high degree of oxido-reductive stress induced by cell lysis in the ER together with the high expression level driven by the strong polyhedrin promoter in baculovirus infected cells often lead to a saturation of the cellular quality control mechanisms, whose role is to ensure that the secretory pathway releases only natively folded proteins (reviewed in [2, 3]). Weaker promoters, e.g., the baculovirus IE1 promoter, have been employed in an attempt to decrease the expression level, thereby resulting in a more efficient processing of complex glycoproteins compared with a standard baculovirus expression system using the polyhedrin promoter [4].

Some viral glycoproteins, such as human immunodeficiency virus (HIV) gp120 and hepatitis virus C (HCV) glycoprotein E2, have been shown to fold slowly, resulting in a longer transit time through the secretory pathway [5, 6]. While the driving forces for these various folding kinetics remain mostly unknown, production of these recombinant proteins seems appropriate in expression systems that induce less ER stress due to lower expression levels than that obtained in baculovirus-based expression systems. Indeed, both HIV gp120 and HCV E2 proteins have been successfully produced in stable *Drosophila* S2 cell transfectants [7, 8]. In at least one reported case (dopamine β-hydroxylase), recombinant protein expression was not successful when using the baculovirus-expression system, but the protein was successfully produced in stable *Drosophila* S2 cell transfectants [9].

### 1.2 Advantages of Expression in Drosophila S2 Cells

Protein expression in stable Drosophila cell transfectants has been primarily performed using Schneider 2 (S2) cells. This cell line is derived from a primary culture of late stage *Drosophila melanogaster* embryos and represents a polyclonal macrophage-like lineage cell line. It was first described over 40 years ago [10] and is now widely used by an increasing number of scientists worldwide. S2 cells are semi-adherent cells, similar to hybridoma cells, growing in a monolayer at low densities and in suspension at high densities. Their optimal growth temperature is 28 °C, but the cells will also grow at room temperature, albeit slower. In contrast to transiently baculovirus-infected cells, stable S2 cell transfectants are healthy and express the protein of interest during cell growth, i.e., they do not suffer the disadvantages implied by the cell lysis mentioned above.

Stable S2 transfectants have a fully functional cellular quality control in the ER (which may especially be crucial for proteins with slow folding kinetics), resulting in the production of a homogeneous recombinant protein. Another major advantage of the S2 based expression system is a stable physiological environment for expression of membrane proteins (reviewed in [11]), a field of research that has been receiving growing attention during the last decade. Finally, once a stable S2 cell transfectant has been generated, the cell line can be easily frozen for long-term storage. Therefore, unlike the baculovirus expression system, expression of recombinant proteins in S2 cells does not require repeated production, titration, and handling of baculoviral stocks for amplification and large-scale expression.

Expression in Drosophila S2 cells is usually driven either by the inducible metallothionein promoter (MT) [12] or by the constitutive Actin 5C promoter (Ac) [13], both of which have been commercialized by Invitrogen in two separate series of vectors (pMT and pAc5.1 vector series, respectively). Comparative studies revealed that the activity of the Actin 5C promoter is approximately 2.5-fold higher than that of the induced MT promoter [14]; however, in particular for membrane proteins, the MT promoter is often preferred because it is inducible. The processing time from DNA cloning to first small scale expression tests in S2 cells can be shortened to less than 5 weeks, while first production in a liter scale is feasible within 7 weeks after transfection. The cells will produce the protein of interest, either continuously or upon induction with divalent cations, and can usually be passaged numerous times in presence of selective antibiotics before a decrease in the expression level is observed (*see* **Note 1**). Since the stable S2 cell transfectants are healthy, exponentially growing cells, almost no cell lysis and release of proteolytic enzymes is observed, therefore minimizing the proteolytic degradation of the protein of interest. In addition, S2 cells can be routinely grown to densities of $3 \times 10^7$ cells/mL, which is about ten times higher than what can be reached with many other insect cell lines. The high cell densities allow for high protein expression levels that render *Drosophila* S2 cells an ideal system for producing high quality recombinant proteins.

Purification of recombinant proteins from stable *Drosophila* S2 cell transfectants naturally depends on the type of recombinant protein and the subcellular compartment in which the recombinant protein is expressed (e.g., secreted into the medium or intracellular). While secreted proteins can easily be purified from serum-free media, additional steps are involved in purifying intracellular proteins to release the protein from the cell. In most cases, purification of the recombinant protein will include affinity chromatography. Numerous affinity tags have been developed in the past decades that allow for efficient affinity purification [15]. The choice of affinity tag will also depend on the subcellular localization

in which the protein is produced. Affinity purification of recombinant proteins has been reviewed elsewhere [15–18], and it is important to appreciate the importance of a suitable purification protocol in order to benefit from the full performance of the *Drosophila* S2 cell expression system.

## 2 Materials

### 2.1 Vectors

1. pMT/BiP vector series (Invitrogen).
2. pAc5.1 vector series (Invitrogen).
3. pCoBlast (Invitrogen) for selection of stable cell lines (*see* **Note 2**).

### 2.2 Medium and Cell Culture

1. *Drosophila* S2 cells (Invitrogen).
2. Cell culture flasks 25 $cm^2$, 75 $cm^2$, and 150 $cm^2$ (Corning).
3. Schneider's complete medium: Schneider medium (Invitrogen) supplemented with 10 % FCS (Invitrogen, heat-inactivated at 56 °C for 30 min), 50 IU/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). This medium is used for transfection, transient expression, and selection (*see* **Note 3**).
4. Blasticidin (Invitrogen) for selection of stable cell lines (*see* **Note 2**).
5. Freezing Container "Mr. Frosty" (Nalgene).
6. Cryo tubes (Nunc).
7. Insect Xpress medium (Lonza) supplemented with 50 IU/mL penicillin and 50 μg/mL streptomycin (Invitrogen). This medium is used for maintaining the culture and large-scale expression using a stable S2 cell line.
8. Inverted light microscope: 4× and 10× objectives.
9. Hemacytometer.

### 2.3 Transfection Reagent

1. Effectene (Qiagen) (*see* **Note 4**).

### 2.4 Large Scale Culture

1. Proculture® 1 L Glass Spinner Flasks (Corning) Magnetic Stirrer (Variomag Biosystem 4).
2. Incubator at 28 °C without $CO_2$ (*see* **Note 5**).

## 3 Methods

### 3.1 Thawing S2 Cells

1. Thaw cells quickly in a 37 °C water bath. When cells have thawed, decontaminate the outside of the vial with 70 % ethanol.

2. Transfer the cells to a centrifuge tube containing 4 mL of Schneider's complete medium and centrifuge at 200 × *g* for 5 min.
3. Resuspend the cell pellet in 5 mL of fresh Schneider's complete medium and transfer the cell suspension to a 25 $cm^2$ flask (*see* **Note 6**).

### 3.2 Passaging of S2 Cells

1. Passaging of S2 cells should be performed when the culture density reaches ~$10^7$ cells/mL.
2. Use a pipette to wash down the surface of the flask to dislodge any adherent cells.
3. Determine the cell concentration by counting.
4. Dilute the cells in Schneider's complete medium to a final concentration of $2 \times 10^6$ cells/mL and seed into a new flask (*see* **Note 7**).

### 3.3 Freezing S2 Cells

1. Freezing of S2 cells should be performed when the culture density reaches ~$10^7$ cells/mL.
2. Freezing of S2 cells can be performed in either *Drosophila* Schneider's complete medium containing FCS, or in serum-free Insect Xpress medium if the cells have already been adapted to this medium. The protocol below applies to both cases.
3. Use a pipette to wash down the surface of the flask to dislodge any adherent cells.
4. Determine the cell concentration by counting.
5. Spin down the cells at 200 × *g* for 5 min. Save the supernatant as "conditioned medium" under sterile conditions and store at 4 °C for later use (*see* **Note 8**).
6. Prepare freezing medium consisting of 10 % DMSO, 45 % fresh medium and 45 % conditioned medium. The total volume of the freezing medium to be prepared will depend on the total cell number (see below).
7. Resuspend the cell pellet gently to a density of >$1.2 \times 10^7$ cells/mL in the freezing medium.
8. Aliquot resuspended cells in 1.5 mL aliquots in cryo vials and freeze them at –80 °C for 24 h in a "Mr. Frosty".
9. Transfer cryovials to liquid nitrogen for long-term storage.

### 3.4 Transfection and Selection of Stable Transfectants

1. Seed $5 \times 10^6$ cells per 25 $cm^2$ flask in 5 mL complete Schneider's complete medium at a density of ~$10^6$ cells/mL.
2. Incubate cells at 28 °C for 24 h (*see* **Note 5**).

#### 3.4.1 Day 0 (Preparation for Transfection)

*3.4.2 Day 1 (Transfection into S2 Cells Using Effectene)*

1. Mix 2.0 μg of your expression plasmid with 0.1 μg of the selection plasmid pCoBlast (*see* **Notes 2** and **9**). Dilute the DNA mixture (minimal DNA concentration of 0.1 μg/μL) with Effectene Buffer EC, to a total volume of 150 μL. Add 16 μL Effectene Enhancer and mix by vortexing for 1 s.
2. Incubate at room temperature (15–25 °C) for 5 min, and then spin down the mixture for a few seconds to remove drops from the top of the tube.
3. Add 20 μL Effectene Transfection Reagent to the DNA–Enhancer mixture. Mix by pipetting up and down five times, or by vortexing for 10 s.
4. Incubate the samples for 15 min at room temperature (15–25 °C) to allow complex formation (*see* **Note 10**).
5. While complex formation takes place, gently aspirate the growth medium from the flask (keep this conditioned medium sterile at 4 °C for later use—*see* **Note 8**), and wash cells once with 2 mL complete Schneider's medium. As cells are only semi-adherent, make sure not to detach the cells by washing, and instead pipette gently onto the flask wall. Add 4 mL fresh complete Schneider's medium to the cells (*see* **Note 11**).
6. Add 1 mL complete Schneider's medium to the tube containing the transfection complexes. Mix by pipetting up and down twice, and immediately add the transfection complexes dropwise onto the cells. Gently swirl the 25 $cm^2$ flask to ensure uniform distribution of the transfection complexes.
7. Incubate cells at 28 °C for 24 h.

*3.4.3 Day 2 (Change of Medium)*

1. IMPORTANT! During the transfection process and selection do not change the culture vessel (*see* **Note 6**).
2. Prepare a mix of fresh complete Schneider's medium (4 mL) and conditioned medium (1 mL).
3. Gently aspirate supernatant into a fresh tube and spin the cells down at 200 *g* for 5 min.
4. Add 2 mL of the medium mix to the 25 $cm^2$ flask (*see* **Note 11**).
5. Resuspend the centrifuged cell pellet in 3 mL of the media mix and add the suspension back to the same 25 $cm^2$ flask.

*3.4.4 Day 4 (Start of the Selection Process)*

Add Blasticidin to a final concentration of 25 μg/mL.

*3.4.5 Day 9 (Change of Medium)*

1. Prepare fresh complete Schneider's medium with Blasticidin (25 μg/mL).
2. Gently aspirate supernatant into a fresh tube and centrifuge at 200 × *g* for 5 min.

3. Add 2 mL of the medium to the 25 $cm^2$ flask (*see* **Note 11**).
4. Resuspend the centrifuged cell pellet in 3 mL of the medium and add the suspension back to the same 25 $cm^2$ flask.

#### 3.4.6 Day 14 (Change of Medium)

1. Prepare fresh complete Schneider's media with Blasticidin (25 μg/mL).
2. Gently aspirate supernatant and centrifuge at 200 × *g* for 5 min.
3. Add 2 mL medium to the 25 $cm^2$ flask (*see* **Note 11**).
4. Resuspend the centrifuged cell pellet in 3 mL medium and add the suspension back to the same 25 $cm^2$ flask.

#### 3.4.7 Day 18 (Amplification and Adaptation to Serum-Free Medium)

1. Gently wash the cells off the flask surface by pipetting up and down and transfer the 5 mL cell suspension to a 75 $cm^2$ flask.
2. Add 5 mL Insect Xpress (serum free) with Blasticidin (25 μg/mL).

#### 3.4.8 Day 21 (Expansion and Adaptation to Serum-Free Medium)

Add 5 mL Insect Xpress with Blasticidin (25 μg/mL).

#### 3.4.9 Day 25 (Expansion)

1. Gently wash the cells off the flask surface and transfer the suspension to a 150 $cm^2$ flask.
2. Add 15 mL Insect Xpress with Blasticidin (25 μg/mL).

#### 3.4.10 Day 28 (Expansion and Freezing Stocks of the Stable Cell Line)

The stable cell line is now established. Use 15 mL of the suspended cells to prepare frozen stocks of the stable cell line as described under Subheading 3.3. Transfer the remaining 15 mL of the resuspended cells to a 150 $cm^2$ flask and add 15 mL Insect Xpress with Blasticidin (25 μg/mL) to start a production culture.

### 3.5 1 L Culture Production

Either continue directly from Subheading 3.4 or thaw a vial of the stable S2 cell transfectant and expand it into a 150 $cm^2$ flask in a total volume of 30 mL Insect Xpress with Blasticidin (25 μg/mL).

#### 3.5.1 Day 1 (Production)

When the cell density in the 150 $cm^2$ flask reaches 10–20 × $10^6$ cells/mL, split the cells 1:6 and add 5 mL cell suspension and 25 mL fresh Insect Xpress with Blasticidin (25 μg/mL)) to each of six 150 $cm^2$ flasks.

#### 3.5.2 Day 8 (Production)

1. Use a pipette to gently wash down the flask surface to dislodge adherent cells and verify that all cells are suspended.
2. Pipette 175 mL cell suspension into a spinner flask (from the six 150 $cm^2$ flasks).
3. Add 225 mL Insect Xpress and grow cells at 28 °C with an agitation rate of 20–40 rpm (*see* **Note 12**).

4. Add 25 mL Insect Xpress with Blasticidin (25 μg/mL) to the remaining 5 mL cell suspension in the 150 $cm^2$ flask to maintain the cell line.

*3.5.3 Day 12 (Production)*

Add 600 mL Insect Xpress (*see* **Note 12**) to the spinner flask.

*3.5.4 Day 15 (Production; Can Be Extended up to Day 18)*

1. Count the cells. When the cell density reaches $12–18 \times 10^6$ cells/mL, induce with 4 μM $CdCl_2$ (*see* **Note 13**).
2. Produce recombinant protein for the optimal induction period (*see* **Note 14**).

*3.5.5 Day 22 (Production)*

Harvest the cells and/or supernatant, and proceed with the appropriate isolation/purification protocol.

## 4 Notes

1. In most cases the stable S2 cell transfectants maintain their expression level for at least 20–30 passages. When a selective pressure due to basal protein expression results in decreased expression levels, this passage number can be as low as 3–7 passages. It is therefore strongly recommended to always freeze stable S2 cell transfectants immediately after transfection in Drosophila Schneider's complete medium.
2. The less expensive and more efficient puromycin (Invivogen), or alternatively hygromycin (Invitrogen), can also be used for selection. However, in this case co-transfection of the cells must include the corresponding plasmid providing resistance to the selection agent such as pCoPuro [19] for puromycin or pCoHygro (Invitrogen) for hygromycin.
3. The vast majority of fetal calf sera (FCS) have proven sufficient to establish stable cell lines expressing the protein of interest. However, compatibility of the FCS with the transfection process should be tested for each lot of FCS independently.
4. Other transfection agents can also be used. We have successfully used Calcium Phosphate (Invitrogen) and Cellfectin (Invitrogen).
5. S2 cells have an optimal growth temperature of 28 °C, but all incubations can also be performed at room temperature, thereby omitting the need for an incubator. However, cells incubated at room temperature will grow slower.
6. IMPORTANT! During transfection and selection keep cells in the same culture vessel. In contrast, always use new flasks or plates for passaging cells for general maintenance.
7. S2 cells do not grow when seeded at densities below $5 \times 10^5$ cells/mL.

8. Conditioned medium contains growth factors that are beneficial for S2 cell growth. As a rule of thumb 20–40 % conditioned medium and 80–60 % fresh medium can be used for passaging. Keep the conditioned medium sterile at 4 °C for later use during cell passaging.
9. Dissolve DNA plasmids in TE or water. Normally a 20:1 (w:w) ratio of expression plasmid to resistance gene plasmid is used. The number of inserted gene copies can be manipulated by varying the ratio of expression to selection plasmids [20]. Depending on the actual expression level as a characteristic of the respective protein of interest, this might play an important role in generation of a stable cell line.
10. Prolonging complex formation for an hour did not decrease the transfection efficiency in our hands.
11. Since S2 cells grow as semi-adherent cells, about 10–30 % of the cells will remain attached to the flask while most cells will stay in the decanted supernatant. Thus, immediately add some medium to the flask after pouring out the supernatant during transfection.
12. Antibiotics are required for each passaging step of stable S2 cell transfectants. However, during amplification for protein production the antibiotics can be omitted.
13. Alternatively, cells can be induced with $CuSO_4$ (500 μM); however, the optimal induction period will be different and needs to be determined empirically (also *see* **Note 14**).
14. The optimal induction period strongly depends on the type and concentration of metal ions utilized for induction (i.e., $CdCl_2$ or $CuSO_4$). Optimal conditions have to be determined in kinetic experiments for each stable S2 cell transfectant.

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# Chapter 18

# Transforming Lepidopteran Insect Cells for Improved Protein Processing and Expression

**Robert L. Harrison and Donald L. Jarvis**

## Abstract

The lepidopteran insect cells used with the baculovirus expression vector system (BEVS) are capable of synthesizing and accurately processing foreign proteins. However, proteins expressed in baculovirus-infected cells often fail to be completely processed, or are not processed in a manner that meets a researcher's needs. This chapter discusses a metabolic engineering approach that addresses this problem. Basically, this approach involves the addition of new or enhancement of existing protein processing functions in established lepidopteran insect cell lines. In addition to improvements in protein processing, this approach has also been used to improve protein expression levels obtained with the BEVS. Methods for engineering cell lines and assessing their properties as improved hosts for the BEVS are detailed. Examples of lepidopteran insect cell lines engineered for improved protein *N*-glycosylation, folding/trafficking, and expression are described in detail.

**Key words** Insect cells, Baculovirus, Baculovirus expression vector system, BEVS, Cell transformation, Genetic engineering, Metabolic engineering, Protein *N*-glycosylation, Glycosyltransferase, Protein folding, Protein trafficking

## 1 Introduction

It is often said that the BEVS has the capacity to produce large quantities of structurally authentic recombinant proteins. A major implication of this statement is that this system has all the co- and post-translational protein processing capabilities of higher eukaryotes. However, this is clearly an overstatement, as it is well known that the protein processing capabilities of established lepidopteran insect cell lines are not identical to those of higher eukaryotes [1].

On one end of the spectrum, some lepidopteran insect cell lines simply lack some of the specific protein processing capabilities of higher eukaryotes. For example, baculovirus-infected Sf-9 cells generally fail to α-amidate recombinant proteins [2–4]. However, such limitations do not always reflect the absence of a specific protein-processing pathway in lepidopteran insect cells, in

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general. In fact, this is true of α-amidation, as at least some baculovirus-infected lepidopteran insect larvae can provide this protein modification [3, 5]. This example illustrates the need to recognize that different types of hosts, including both established lepidopteran insect cell lines and lepidopteran insect larvae, can have different protein processing capabilities, which is an important consideration in choosing an appropriate host for recombinant protein production.

Beyond the absolute presence or absence of a specific protein processing capability, one also must consider the efficiency of protein processing in the BEVS. There are published examples of inefficient proteolytic processing [6], *N*-glycosylation [7], *O*-glycosylation [8], phosphorylation [9], and acylation [10, 11] of recombinant proteins in baculovirus-infected insect cells. It has been widely speculated that these processing inefficiencies might reflect the inability of the host protein processing machinery to accommodate the massive amounts of recombinant protein that can be produced during baculovirus infection. Moreover, the potential saturation of these pathways might be exacerbated by repression of host gene expression, which occurs during baculovirus infection [12, 13], and by deleterious effects of baculovirus infection on host protein processing pathways [14, 15]. These factors also might impair the efficiency of protein folding and reduce protein solubility in baculovirus-infected cells, thereby reducing functional protein yields.

Finally, it must be recognized that the specific nature of any given protein-processing pathway is not necessarily identical in insect cells and higher eukaryotes. For example, differences in the structures of the *N*- and *O*-glycans produced by insect and mammalian cells provided an early indication that there are fundamental differences in their protein glycosylation pathways, a fact that is now well established in the literature [1, 16–19].

One way to address the types of protein processing problems described above is to use metabolic engineering to extend the functional capabilities of insect protein processing pathways. In essence, this involves introducing higher eukaryotic functions into lepidopteran insect cells using the genetic transformation approach described in Chapter 16. The resulting cell lines are designed to serve as improved hosts for the BEVS with the ability to produce more accurately and/or efficiently processed recombinant proteins. In this chapter, we will describe procedures for the production and evaluation of lepidopteran insect cell lines engineered in this fashion. These procedures involve (a) constructing constitutive expression plasmids encoding the desired protein processing functions, (b) using the resulting constructs to produce stably transformed lepidopteran insect cell lines, and (c) assessing the relevant properties of the new cell lines with respect to their application as improved hosts for the BEVS.

## 2 Materials

1. Cell lines: Sf-9 (ATCC: #CRL-1711) can be obtained from the American Type Culture Collection (Manassas, VA 20108; www.atcc.org) or from Life Technologies (Carlsbad, CA; www.lifetechnologies.com).
2. Cell media: Grace's Insect Medium, Hink's TNM-FH medium, and Pluronic® F-68 are available from Sigma-Aldrich Corporation (St. Louis, MO; www.sigma-aldrich.com).
3. Plastics: Falcon and Corning flasks, Petri dishes, and multiwell plates for cell culture are available from Fisher Scientific (Pittsburgh, PA; www.fishersci.com).
4. Hemacytometer and cell counters: available form Fisher Scientific.
5. Fetal bovine serum is available from Life Technologies.
6. Conventional cell culture antibiotics: Amphotericin B (Fungizone®) and gentamicin can be obtained from Life Technologies.
7. Antibiotics for selection of transformed cells: G418 (Geneticin®), hygromycin B, Zeocin™, and blasticidin S are available from Life Technologies.
8. Enzymes for constructing expression plasmids: Restriction endonucleases, calf intestinal alkaline phosphatase, DNA polymerase I (Keno fragment), and T4 DNA polymerase can be obtained from New England Biolabs (Ipswich, MA; www.neb.com).
9. DeLong flasks (flasks with long necks designed to reduce splashing and accommodate plastic or stainless steel closures) are available from Fisher Scientific (Pittsburgh, PA; www.fishersci.com).
10. SeaPlaque low melting point agarose (catalog #50101) from Lonza (Basel, Switzerland; www.lonza.com) for virus plaque assays.
11. 2× TNM-FH can be prepared as previously described [20] or purchased from a commercial vendor such as Sigma-Aldrich (St. Louis, MO; www.sigmaaldrich.com).
12. Plasmids: The pIEHR series of expression vectors, the dual gene expression vector pDIE1HR1 (Fig. 1), the pDIE1-(fluorescent protein)-TOPO.4 series of expression vectors (Fig. 1), and the selection plasmids pIE1Neo and pIE1Hygro are available from Dr. Donald Jarvis upon request. A series of vectors that use the same hr5-ie-1 enhancer-promoter arrangement (the pIEx series) can be purchased from EMD Millipore (Billerica, MA; www.emdmillipore.com). In addition, expression plasmids (the

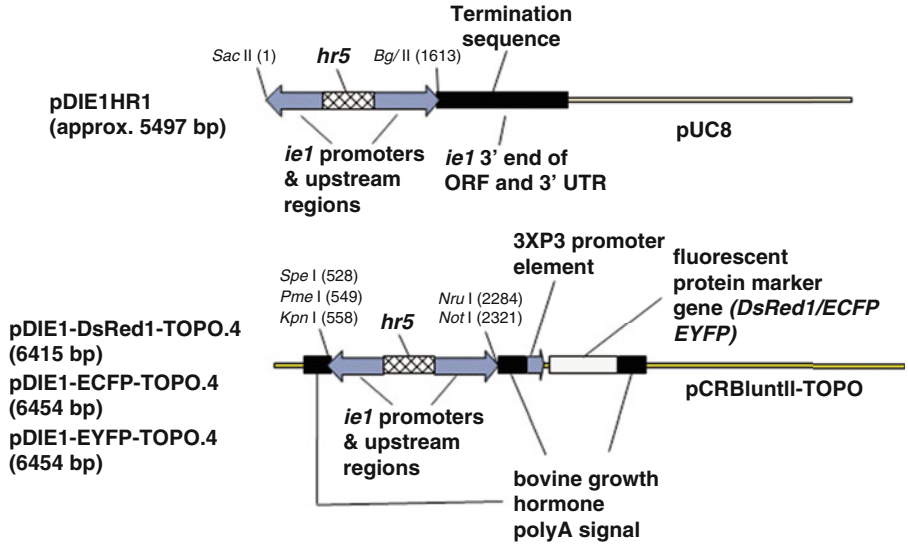


**Fig. 1** Features of pDIE1HR1 and the pDIE1-(fluorescent protein)-TOPO.4 plasmid vectors for stable transformation and constitutive expression of genes in lepidopteran insect cell lines. These plasmids contain a *hr5* enhancer and two back-to-back *ie-1* promoters, as indicated. pDIE1HR1 includes a transcriptional termination signal downstream of the right-hand *ie-1* promoter, but not downstream of the left-hand promoter. The pDIE1-(fluorescent protein)-TOPO.4 plasmids contain bovine growth hormone polyadenylation signals downstream of both *ie-1* promoters and the fluorescent protein coding sequence. A *D. melanogaster 3xP3* promoter construct drives expression of the fluorescent protein genes. Restriction endonuclease sites unique to each vector are available for inserting genes of interest downstream of the *ie-1* promoter regions. The *ie-1* translational initiation codons downstream of both *ie-1* promoters have been *deleted* in both constructs

pIB and pIZ series) that carry drug resistance genes for blasticidin S and Zeocin™ can be purchased from Life Technologies (Carlsbad, CA; www.lifetechnologies.com).

## 3 Methods

### 3.1 Transformation, Selection, and Isolation of Transformed Clones

Lepidopteran insect cell lines engineered to have improved protein processing capabilities can be isolated by following the procedures outlined in Chapter 16. Generally, this involves the introduction of constitutively expressible genes encoding missing or suboptimal protein processing functions into the genome of an established lepidopteran insect cell line. Depending on the nature of the desired protein modification, it might be necessary to introduce more than one gene. For the insertion of two genes into the cellular genome in a single transformation event, we have constructed the expression plasmid designated pDIE1HR1 [21]. This plasmid contains a bidirectional enhancer/promoter element consisting of a single copy of the baculovirus *hr5* enhancer flanked by two copies of the *ie-1* promoter in opposite orientations (Fig. 1).

The back-to-back *ie-1* promoters in this vector provide constitutive expression of both genes in Sf-9 and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5, commercially available from Invitrogen as High Five™) cells [21, 22]. More recently, we have constructed plasmids designated pDIE1-DsRed1-TOPO.4, pDIE1-EYFP-TOPO.4, and pDIE1-ECFP-TOPO.4, which are similar to pDIE1HR1, but also contain a fluorescent marker gene (Discoma red fluorescent protein, or DsRed1; enhanced yellow fluorescent protein, or EYFP; and enhanced cyan fluorescent protein, or ECFP) under the control of the *Drosophila melanogaster* eye-specific promoter, 3xP3 (Fig. 1) [23]. Rather unexpectedly, we found that the 3xP3 promoter drives visible levels of fluorescent marker gene expression in Sf-9 cells transfected with these vectors and their derivatives (Harrison and Jarvis, unpublished observations).

Several approaches can be used to insert more than two genes into the genome of an established lepidopteran insect cell line, which might be necessary to engineer more elaborate protein processing pathways, such as protein glycosylation pathways. One approach is to transfect the cells with multiple plasmids carrying individual genes of interest, and then screen the resulting transformants for each of the required functions (*see* **Note 1**). Another is to introduce the individual genes in a sequential series of separate transformation experiments. This latter approach can be used to extend a pathway one function at a time, until one obtains a transformed cell line with the ability to provide the desired protein modification. The sequential approach also can be used to create cell lines that produce otherwise unavailable protein processing intermediates, which can be used for structural and functional studies. The antibiotics that have been used to select lepidopteran insect cell transformants include G418, hygromycin B, puromycin, Zeocin™, and blasticidin S. Each has a different mechanism of action and theoretically can be used for the sequential transformation approach (*see* **Note 2**). Indeed, we have sequentially transformed Sf-9 cells using G418 for the first, hygromycin B for the second, and Zeocin™ for the third round of selection [22, 24, 25].

### 3.2 Screening for Integration and Expression of New Protein Processing Gene(s)

Once the appropriate genetic constructs have been introduced and antibiotic-resistant lepidopteran insect cell subclones isolated, they must be screened for the presence and ability to express the new processing gene(s). This can be accomplished using Southern blotting, PCR, RNA dot blot, northern blotting, and/or RNAse protection assays, as described in Chapter 16. In addition, because these transformed subclones are to be used as improved hosts for baculovirus-mediated recombinant protein production, one must examine other properties related to their ability to serve this host function.

### 3.3 Assessing Growth Properties of Stably Transformed Cells

The introduction and constitutive expression of new protein processing genes might be expected to alter the overall metabolism of a lepidopteran insect cell line, which might alter its growth rate, optimal seeding density, and/or maximal achievable density. One also can imagine other phenotypic changes, such as differences in cell size, morphology, and/or the ability to grow in suspension or as an adherent culture, stemming from the alteration of protein processing pathways. The following is one protocol that can be used to measure the growth rate and viability of a newly transformed Sf-9 derivative in suspension culture (*see* **Note 3** and Chapter 1).

1. Seed 125 mL DeLong flasks with a total volume of 50 mL of the parental and transformed Sf-9 cells at a density of $0.6 \times 10^6$ cells/mL in complete TNM-FH. If the functionality of the protein processing pathway engineered in the transformed cells requires additional medium supplements, then set up separate cultures with medium containing those supplements.
2. Incubate the cultures in a shaking incubator adjusted to 125 rpm and 28 °C.
3. At various times after seeding (generally at 24 h intervals), remove triplicate 1-mL samples from each culture. Add 0.1 mL of a 0.4 % solution of trypan blue to each sample and mix.
4. Transfer a small aliquot from each sample to a hemacytometer and perform a viable cell count to measure the average cell concentrations and viabilities (percentage of cells that exclude trypan blue). *See* Chapter 1 for details about how these data can be used to obtain a growth curve and a population doubling time (PDT), as well as alternative methods for obtaining cell densities.
5. A transformed cell line PDT that is comparable to that of wild-type cells is a reasonable goal. However, investigators should consider their overall objectives when assessing this parameter.

### 3.4 Assessing the Susceptibility of Stably Transformed Cells to Baculovirus Infection

A stably transformed lepidopteran insect cell line with altered protein processing capabilities should have structurally altered endogenous proteins, which might adversely influence their ability to serve as hosts for the BEVS. The most obvious potential problem would be an alteration in protein processing pathways that would change the structures of cell surface proteins, as this could decrease or eliminate baculovirus binding, penetration, assembly, and/or release. Clearly, any transformed cell line with a diminished capacity to serve as a baculovirus host would be unacceptable. Thus, it is important to carefully examine the host function(s) of any lepidopteran insect cell line that has been transformed to improve its protein processing capabilities. The following is a protocol for a

one-step baculovirus growth experiment that can be used to assess overall host function.

1. Seed 250 mL DeLong flasks with a total volume of 100 mL of the parental and transformed Sf-9 cells at a density of $0.6 \times 10^6$ cells/mL in complete TNM-FH. If the functionality of the protein processing pathway engineered in the transformed insect cell line requires additional medium supplements, then set up separate cultures with medium containing those supplements.
2. Incubate the cultures in a shaking incubator adjusted to 125 rpm and 28 °C. Allow the cells to grow until they reach a concentration of approximately $1.25 \times 10^6$ cells/mL.
3. Pellet the cells from each culture by low-speed centrifugation ($500 \times g$ for 3 min).
4. Resuspend the cell pellets in a small volume of complete TNM-FH. Infect at an MOI of 5 by adding the appropriate volume of a wild-type or recombinant baculovirus. The final cell density after the addition of inoculum should be approximately $10^7$ cells/mL. Incubate each culture on a rocking platform for 1 h at 28 °C.
5. Re-pellet the cells as in **step 2** and wash three times with complete TNM-FH.
6. Resuspend the cell pellets in 100 mL complete TNM-FH and transfer to a 250-mL DeLong flask. Incubate each infected cell culture at 125 rpm and 28 °C and harvest triplicate 1 mL samples of the infections every 12–24 h starting immediately after the 1 h virus attachment period. Pellet the cells in each sample, harvest the supernatants, and titer the virus (*see* Chapter 4, 5, 10, 11, and 22 for various methods of titering baculoviruses).
7. A budded virus titer from infected transformed cell lines that is comparable to that of infected wild-type cells is a reasonable goal. However, investigators should consider their overall objectives when assessing this parameter.

It is conceivable that the baculovirus virion proteins required for virus attachment and entry may be modified in transformed cell lines in ways that diminish or eliminate the ability of progeny budded virus from infected transformed cells to establish a productive infection of Sf-9 cells during a plaque assay. The formation of polyhedra in infected cells is also an indicator of the efficiency of virus infection and replication, and it can be informative to observe the relative levels of polyhedra that appear in infected cells when assessing the infectability of transformed cell lines. If desired, polyhedra can be easily isolated from infected cells and quantified by the following protocol, modified from O'Reilly et al. [26].

8. Suspend the cell pellets from **step 6** in 1 mL 0.5 % SDS.
9. Pellet the polyhedra by centrifugation at 5000 × *g* for 5 min.
10. Resuspend the polyhedra in 1 mL 0.5 M NaCl. Re-pellet the polyhedra.
11. Resuspend the polyhedra in 0.5 mL $dH_2O$ and count with a hemacytometer.
12. A quantity of polyhedra produced in infected transformed cell lines that is comparable to polyhedra quantity in wild-type cells is a reasonable goal. However, investigators should consider their overall objectives when assessing this parameter.

### 3.5 Assessing Baculovirus-Mediated Foreign Gene Expression in Stably Transformed Cells

A reduction in the expression levels that can be achieved with the BEVS is another potentially deleterious effect of altering the processing of endogenous lepidopteran insect cell proteins. The one-step growth curve experiment described above can predict, to some extent, how well a recombinant virus might express a foreign protein of interest in a newly transformed insect cell line. This is because the foreign gene of interest is expressed under the transcriptional control of the polyhedrin promoter in most baculovirus expression vectors. Hence, the relative quantities of polyhedra produced by parental and transformed Sf-9 cells infected with the wild-type baculovirus will reflect the relative levels of polyhedrin-mediated gene expression in the different cell lines.

However, it is important to recognize that multiple factors influence polyhedra production and that recombinant protein production levels by the BEVS are highly protein-specific. Accordingly, it is important to directly examine the ability of any newly transformed insect cell line to produce the specific recombinant protein of interest. Moreover, it is important to recognize that the relative expression levels achieved with that specific protein might or might not reflect the general expression levels that can be achieved with the new cell line.

To compare recombinant protein production levels, separately infect parental and newly transformed insect cell cultures with a specific baculovirus expression vector, as described under Subheading 3.4. Harvest aliquots of the infected cell cultures daily from 1 to 5 days post-infection and measure the relative amounts of recombinant protein present in each aliquot. Assays that can provide quantitative or semiquantitative comparisons of recombinant protein levels include gel electrophoresis with protein staining, immunoblotting, ELISA tests, and enzyme activity assays, among others.

### 3.6 Assessing Protein Processing by Transformed Cells

It is possible to produce a stably transformed insect cell line that contains and expresses a new protein processing gene, but fails to process a BEVS-encoded recombinant protein in the expected fashion. This could result from the inability of a protein-processing

enzyme to accumulate in the appropriate subcellular compartment and/or from the absence of various cofactors required for the enzyme to perform its function in vivo.

For these reasons, one should use a model protein to assess the ability of a new insect cell line engineered to have a new protein processing function to actually perform that function. The methods used for this experiment will obviously depend upon the function being assessed.

## 3.7 Examples of Stable Transformation for Improved Protein Processing

Due to its important influence on protein function, stability, and pharmacokinetics, our efforts to transform insect cells to improve their protein processing capabilities have focused on the protein *N*-glycosylation pathway [27, 28]. In general, these efforts were designed to isolate stably transformed insect cell lines that can produce recombinant glycoproteins with mammalian-like *N*-glycans. More specifically, we have focused on the inability of established lepidopteran insect cell lines to sialylate recombinant *N*-glycoproteins. One reason this is important is that terminal sialylation blocks the clearance of glycoproteins by carbohydrate-specific receptors in mammals [29]. Thus, therapeutic glycoproteins produced with the baculovirus-insect cell system, which typically lack terminal sialic acids, are unlikely to be efficacious because they will be rapidly cleared from the mammalian bloodstream after injection [30].

### 3.7.1 Sf-9 and Sf-21 Cells Transformed for Altered Protein N-Glycosylation

The early steps in protein *N*-glycosylation in both insect and mammalian cells involve transfer of the same precursor oligosaccharide to an asparagine residue in a nascent polypeptide, followed by trimming of the new side chain by the same or a highly similar set of glycosidases [31]. This results in the production of a common intermediate in both cell types. In mammalian cells, this intermediate is elongated by a series of glycosyltransferases to produce "hybrid" or "complex" *N*-glycans, which often have terminal sialic acid residues (Fig. 2, right side). In contrast, glycoproteins produced by established lepidopteran insect cell lines typically contain "high mannose" and/or "paucimannose" *N*-glycans with no terminal sialic acids (Fig. 2, left side). We should note that it is now quite clear that at least some insects have the endogenous capacity to produce complex, terminally sialylated *N*-glycans like those found on mammalian glycoproteins [18, 32–34]. However, this appears to be a limited capacity that has only been demonstrated clearly in Drosophila and is restricted, even in the fly, to a small number of cells within the central nervous system [35]. We also recognize that there are scattered reports claiming that lepidopteran insect cell lines can produce sialylated glycoproteins [18]. However, a recent study revealed that at least some of those, involving the use of secreted alkaline phosphatase as a model protein, are confounded by contamination of the "purified" model glycoprotein with serum sialoglycoproteins from the insect cell growth medium

used for the experiments [35]. In fact, there are no confirmed reports of recombinant *N*-glycoprotein sialylation by baculovirus-infected lepidopteran insect cell lines. Thus, the major processed *N*-glycans found on recombinant glycoproteins produced by baculovirus-infected insects and insect cell lines are highly trimmed, paucimannose structures, not complex, terminally sialylated structures like those produced by mammalian cells (Fig. 2).

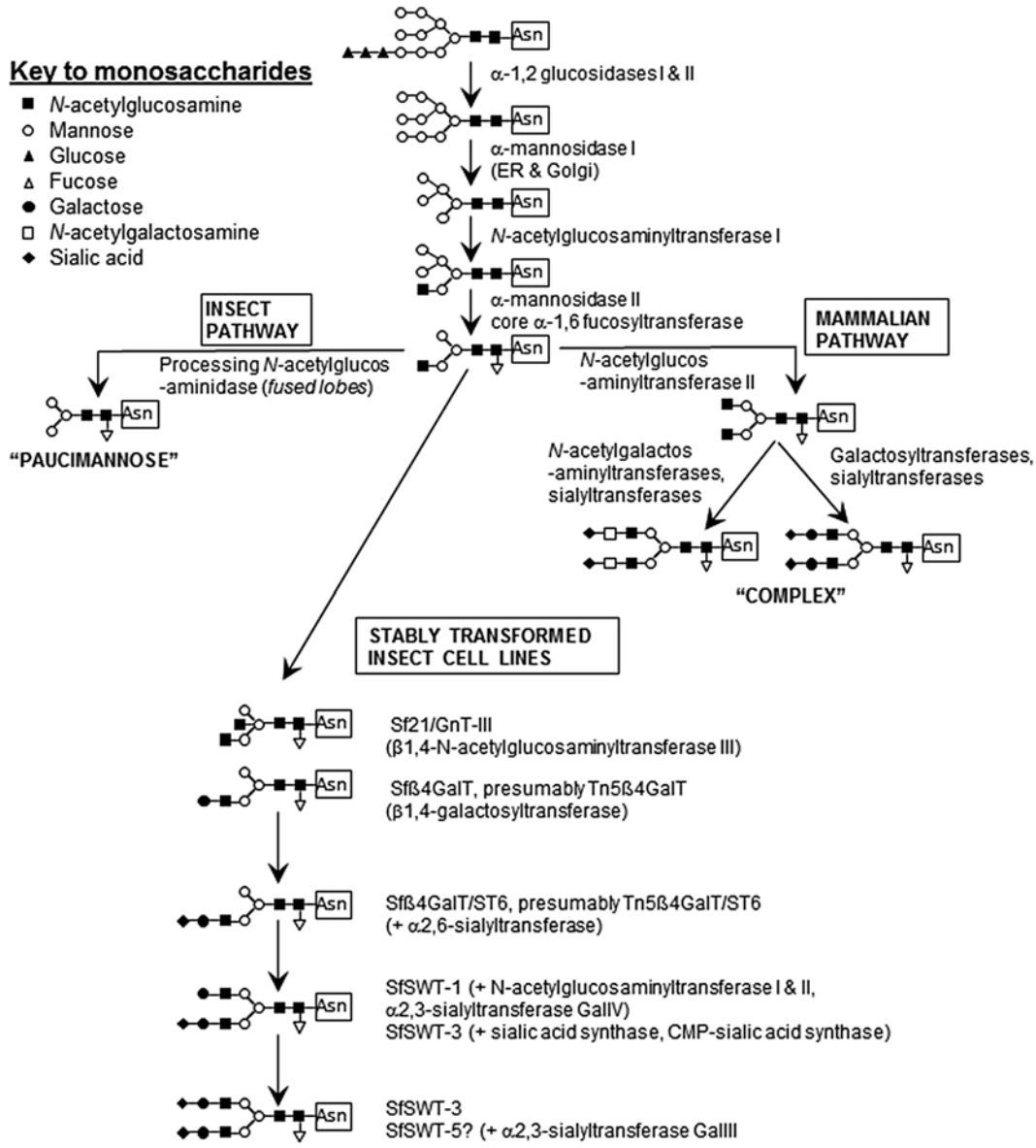


**Fig. 2** *N*-glycosylation pathway in insects and mammals, and the *N*-glycans formed in transformed cell lines. Modified from ref. 27 with permission from Elsevier

The first insect cell line to be genetically transformed for the purpose of extending its *N*-glycan processing capabilities was Sfβ4GalT [36]. This cell line consisted of Sf-9 cells transformed with an immediate early expression plasmid [37] encoding bovine β1,4-galactosyltransferase (B4GALT1) under the transcriptional control of a baculovirus *ie-1* promoter. This is the enzyme that normally transfers galactose to terminal *N*-acetylglucosamine residues during the elongation phase of mammalian *N*-glycan processing. Lectin blotting assays showed that the baculovirus envelope fusion glycoprotein, gp64, acquired terminal, ß-linked galactose residues when produced during baculovirus infection of Sfβ4GalT, but not Sf-9 cells. These results showed that the endogenous Sf-9 cell protein *N*-glycosylation pathway could be extended by the introduction of a constitutively expressible mammalian glycosyltransferase gene.

Subsequently, Sfβ4GalT cells were super-transformed with an immediate early expression plasmid encoding rat α2,6-sialyltransferase I (ST6GAL1) to produce a cell line designated Sfβ4GalT/ST6 [24]. This new cell line produced gp64 and an additional model glycoprotein, GST-SfManI, with terminally sialylated *N*-glycans. Transformation of Tn-5 cells with the same mammalian B4GALT1 and ST6GAL1 genes using the dual immediate early expression plasmid described above also yielded transformed insect cells lines with the ability to produce terminally galactosylated and sialylated glycoproteins [21].

Lectin blotting is a rapid and valid way to evaluate the structures of *N*-glycans when used in a proper and appropriately controlled fashion (*see* **Note 4**). However, it is an indirect method and provides only compositional information about glycoprotein glycan structure. Thus, it is important to confirm and extend any conclusions based on lectin blotting alone using more direct methods. We have used HPLC and mass spectroscopy for this purpose and the results of these additional analyses revealed that the *N*-glycans synthesized by Sfβ4GalT and Sfβ4GalT/ST6 cells had, indeed, acquired terminal galactose and sialic acid residues, respectively, but only on one branch [25, 38]. While the lower (α3) branch of the *N*-glycans produced by these cells terminated in galactose or sialic acid, the upper (α6) branch terminated in mannose. In context of the well-established nature of the mammalian *N*-glycan processing pathway [39] and the prior observations of Altmann and coworkers [40], this result clearly indicated that Sf-9 cells had insufficient endogenous *N*-acetylglucosaminyltransferase II (MGAT2) activity to initiate elongation of the upper branch. Thus, we went on to isolate a cell line designed to produce *N*-glycans with truly "complex", biantennary *N*-glycans by transforming Sfβ4GalT cells with immediate early expression plasmids encoding a more comprehensive selection of mammalian glycosyltransferase genes, including *N*-acetylglucosaminyltransferase I (MGAT1),

MGAT2, B4GALT1, ST6GAL1, and α2,3-sialyltransferase IV (ST3GAL4). The resulting cell line, designated SfSWT-1, produced complex, biantennary *N*-glycans in which the α3 branch terminated with sialic acid and the α6 branch terminated with galactose [25].

Interestingly, Sfβ4GalT/ST6 and SfSWT-1 cells were able to produce terminally sialylated glycoproteins even though Sf-9 cells have no detectable CMP-sialic acid, the donor substrate required for glycoprotein sialylation [41, 42]. Further work suggested that sialic acid was somehow being salvaged from high molecular weight components of the fetal bovine serum, including mammalian sialoglycoproteins, in the cell medium [43]. To produce a cell line that could sialylate glycoproteins in the absence of serum, SfSWT-1 cells were transformed with immediate early expression plasmids encoding two mammalian enzymes involved in CMP-sialic acid production, sialic acid synthase (SAS) and CMP-sialic acid synthetase (CMAS) [22]. The resulting cell line, designated SfSWT-3, was able to produce complex, terminally sialylated *N*-glycans when cultured in serum-free medium supplemented with *N*-acetylmannosamine, a precursor for sialic acid biosynthesis.

Structural analyses have shown that the predominant *N*-glycans produced by both SfSWT-1 and SfSWT-3 cells are α2,6-sialylated on the α3 branch. SfSWT-3 cells produce an additional, but very minor, *N*-glycan fraction that appears to have terminal sialic acid residues on both branches. These results, together with the results of recent transient expression assays designed to measure the induction of α2,3-sialyltransferase activity by the immediate early expression plasmid encoding ST3GAL4 (Harrison & Jarvis, unpublished data), suggest that this plasmid cannot induce this activity in Sf- cells. Hence, we produced another transformed insect cell line, SfSWT-5 [44], using three *piggyBac* transposon-based dual-expression vectors carrying five of the seven mammalian glycan processing genes present in SfSWT-3—namely, MGAT2, B4GALT1, ST6GAL1, SAS, and CMAS—and a sixth gene, murine α2,3-sialyltransferase III (ST3GAL3) [45]. Unlike ST3GAL4, ST3GAL3 can induce α2,3-sialyltransferase activity in transfected Sf-9 cells [23]. SfSWT-5 cells also have α2,3-sialyltransferase activity [44], but the presence of terminal sialic acids on the α6 branch of recombinant glycoproteins expressed in these cells has yet to be confirmed.

The paucimannose-type *N*-glycans found on insect cell-derived glycoproteins (Fig. 2) are directly produced by the action of a processing β-N-acetylglucosaminidase encoded by orthologs of the *D. melanogaster fused lobes* (*fdl*) gene [46, 47]. By removing the terminal *N*-acetylglucosamine residue from the α3 branch of the core glycan, FDL antagonizes the impact of MGAT1 and MGAT2 [48] and reduces *N*-glycan elongation efficiencies in SfSWT-1 and -3 cells. Hence, another approach

that can theoretically be used to alter *N*-glycan processing in insect cells is to block or eliminate FDL activity. Towards this end, Okada and coworkers [49] transformed Sf-21 cells with a constitutive expression vector encoding rat β1,4-*N*-acetylglucosaminyltransferase III (MGAT3) [50] to produce a subclone designated Sf21/GnT-III. MGAT3 catalyzes the addition of a bisecting *N*-acetylglucosamine residue to the β1,4-mannose residue in the core *N*-glycan structure (Fig. 2). Terminal *N*-acetylglucosamine residues of bisected *N*-glycans are generally resistant to cleavage. Thus, it was expected that this MGAT3-mediated *N*-glycan modification would prevent the cleavage removal of terminal *N*-acetylglucosamine residues and block the formation of paucimannosidic structures by Sf-21 cells. This expectation was upheld by an analysis of the *N*-glycans from total cellular glycoproteins and a model glycoprotein expressed with a baculovirus vector, which showed that the proportion of core glycans with a terminal *N*-acetylglucosamine residue was significantly higher in Sf21/GnT-III than in the parental Sf-21 cells [49]. In a separate study, Geisler and coworkers [47] cloned the *fdl* ortholog (*Sf-fdl*) from Sf-9 cells and used the sequence to produce an Sf-9 derivative transformed with a constitutive expression vector encoding a double-stranded RNA trigger designed to suppress *Sf-fdl* expression via RNA interference (RNAi) [51]. The transformed cells had significantly less FDL activity than Sf-9 cells, but the impact of FDL suppression on the structures of core *N*-glycans was not assessed.

It is conceivable that the introduction and expression of genes that alter the biochemical pathways in insect cells associated with glycan processing imposes a metabolic burden on the cells that could lead to suboptimal growth properties, loss of the introduced genes, and reduced capacity to support infection, replication, and recombinant protein expression by baculovirus vectors. To address this issue, the SfSWT-5 cell line was generated with vectors in which the glycan processing genes were placed under the control of a tetracycline-inducible promoter construct consisting of back-to-back cytomegalovirus promoters separated by a tetracycline operator element [23, 44]. These cells were also transformed with a vector that constitutively expresses a tetracycline-sensitive repressor of the tetracycline operator element, so that the glycan processing genes in SfSWT-5 could be induced by the addition of doxycycline, a synthetic tetracycline analog. No significant changes in growth properties or viability of SfSWT-5 cells were observed when expression of the glycan processing genes was induced with doxycycline. Furthermore, there were no major differences in the growth properties of SfSWT-5 cells continuously cultured in the presence or absence of doxycycline for >300 passages (1.5 years). These results are consistent with previous observations of the growth properties of the SfSWT-1 and SfSWT-3 cell lines [22].

SfSWT-5 cells also maintained functional expression of the glycan processing genes after >300 passages in the presence of doxycycline, as assessed by staining of the cells with a sialic acid-binding lectin. One model glycoprotein was expressed at similar levels after baculovirus infection of Sf-9, SfSWT-1, SfSWT-3, and SfSWT-5 cells, although there were some differences in expression kinetics [22, 43]. These results indicate that the transformed cell lines expressing glycan processing genes were able to support baculovirus infection at levels similar to those of the parental Sf-9 cells. This was somewhat surprising, as Sfβ4GalT/ST6, SfSWT-1, SfSWT-3, and SfSWT-5 cells should terminally sialylate endogenous membrane glycoproteins, which would dramatically alter the surfaces of these cells. This might be expected to inhibit virion attachment, penetration, assembly, and/or release, as discussed above. Instead, it appears that engineering the glycan-processing pathways of insect cells with mammalian genes has little to no discernible effect on the capability of the cells to support recombinant glycoprotein production using baculovirus expression vectors.

### *3.7.2 Sf-9 and Tn-5 Cells Transformed for Improved Protein Folding*

Deficiencies in protein folding processes might account, at least in part, for the general inability of baculovirus-infected insect cells to produce large quantities of functional secretory pathway products (*see* Chapter 16). This idea has been supported by the results of a number of studies on baculovirus expression of secretory pathway proteins, which showed functional protein yields were higher when insect cells were coinfected with a baculovirus vector expressing the protein of interest and a second vector expressing a molecular chaperone or foldase [52–56].

In an effort to improve protein folding and enhance recombinant protein secretion from insect cells, Kato and coworkers [57] produced stably transformed Tn-5 cell clones that constitutively produced either human connexin or human calreticulin. These lectin chaperones bind nascent glycoproteins in the ER and prevent aggregation, retain folding intermediates, and promote disulfide bond formation and isomerization [58]. Constitutive expression of connexin or calreticulin increased the amounts of extracellular GFP-tagged β1,3-*N*-acetylglucosaminyltransferase 2 ($GFP_{uv}$-β3GnT2) activity produced by a baculovirus vector and by cells stably transformed with the $GFP_{uv}$-β3GnT2 gene. Increases in extracellular β3GnT2 activity were more consistently observed in clones stably transformed with human calreticulin [57].

Lenhard and Reiländer [59] addressed the protein folding issue in baculovirus-infected Sf-9 cells by transforming the cells with a gene encoding the NinaA protein of *Drosophila melanogaster*. NinaA is a type I membrane protein with homology to cyclophilins, which have peptidyl-prolyl *cis-trans* isomerase activity and are involved in a number of functions, including protein folding [60]. NinaA has been shown to be required for the accumulation and trafficking of the *D. melanogaster* rhodopsin, Rh1 [61, 62].

The resulting transformed Sf-9 derivative, designated Sfn, constitutively expressed NinaA at levels that were detectable by immunoblotting analysis and grew more slowly than Sf-9 cells, accumulating to only half the density of the parental cells in suspension culture. Sfn and Sf-9 cells produced about the same amounts of three different G-protein coupled receptors upon infection with recombinant baculoviruses. However, baculovirus-infected Sfn cells expressing the human dopamine transporter exhibited a larger (≥5-fold) increase in dopamine uptake than Sf-9 cells infected with the same virus. Dopamine uptake by Sfn cells was reduced 40 % by a cyclophilin peptidyl-prolyl *cis-trans* isomerase inhibitor, while dopamine uptake by Sf-9 cells was unaffected by treatment with the same inhibitor. The authors concluded that expression of NinaA was responsible for the higher levels of functional, plasma membrane-localized dopamine transporter expression observed in Sfn cells.

### 3.7.3 Sf-9 Cells Transformed for Increased Recombinant Protein Expression

Healthy insect cell cultures are critical for efficient baculovirus infection and replication. Culture conditions that adversely affect insect cell health and/or stressors that affect the ability of insect cells to support viral replication and gene expression during infection may reduce the yield of recombinant protein obtained in the BEVS. Thus, one could conceivably use stable transformation to produce insect cell lines that are resistant to stressful cell culture conditions or that are more efficient hosts for baculovirus infection and replication.

Lin and coworkers [63] pursued this strategy by creating a stably transformed Sf-9 cell line that constitutively expresses a baculovirus P35 protein. Baculovirus infection normally triggers a pathway of molecular events leading to apoptosis, or programmed cell death, in host cells [64, 65]. Apoptosis is a defensive response to infection, but baculoviruses encode a variety of proteins, including P35, that block the apoptotic pathway [64, 65]. Thus, as expected, the transformed Sf-9 cells constitutively expressing P35 were resistant to apoptosis triggered by actinomycin D or nutrient stress simulated by incubation in phosphate-buffered saline, as indicated by relatively higher levels of survival [63]. In addition, infection of these transformed clones with baculovirus vectors encoding either β-galactosidase (an intracellular protein) or secreted alkaline protease (SEAP, a secretory pathway protein) yielded higher levels of both proteins than the parental Sf-9 cells, although this trend was not observed with every clone or at every time post-infection [63]. Nevertheless, one clone produced SEAP at fourfold higher levels than Sf-9 cells after 7–9 days of baculovirus infection.

Another group also found they could improve recombinant protein expression levels by metabolically engineering Sf-9 cells to block apoptosis [66]. In this case, the approach was to transform

Sf-9 cells with an RNA hairpin construct designed to trigger RNAi-mediated suppression of the cellular caspase gene, *Sf caspase-1* [67]. The transformed clones had lower steady-state levels of *Sf caspase-1* mRNA than the parental Sf-9 cells, which correlated with reduced apoptosis and higher cell viabilities after exposure to ultraviolet light (another apoptosis inducer) or infection with a recombinant baculovirus encoding *Sf caspase-1*. Finally, the transformed cells produced 1.5- to 2.5-fold more recombinant luciferase (an intracellular protein) and SEAP than the parental Sf-9 cells after being infected with baculovirus vectors encoding these proteins [66].

Analogous results have been obtained by transforming Sf-9 cells with members of the polydnavirus *vankyrin* gene family [68]. These genes encode proteins with sequence similarity to ankyrins or inhibitor κB proteins that block the NF- κB signal transduction pathway involved in the activation of the insect immune response [69]. Interestingly, Sf-9 cells infected with recombinant baculoviruses expressing the *vankyrin* genes *P-vank-1* and *$I^2$-vank-3* from the polydnavirus *Campoletis sonorensis* ichnovirus had increased longevity and delayed cell lysis, relative to Sf-9 cells infected with a wild-type baculovirus [70]. Moreover, baculovirus dual expression vectors encoding both yellow fluorescent protein (YFP) and P-vank-1 produced 16-fold more YFP than cells infected with a baculovirus encoding YFP, alone [68]. To assess if constitutive *vankyrin* gene expression could improve baculovirus vector-mediated protein expression, Fath-Goodin and coworkers transformed Sf-9 cells with either *P-vank-1* or *$I^2$-vank-3* expression constructs [68]. The transformed cells produced eightfold more YFP and augmented secretion of the polydnavirus VHv1.1 protein when infected with recombinant baculoviruses encoding these proteins, as compared to parental Sf-9 cell controls. The increase in YFP expression correlated with significantly higher longevity among *P-vank-1*-transformed Sf-9 cells infected with the YFP-expressing baculovirus [68, 71]. *P-vank-1*-transformed Sf-9 cells were resistant to apoptosis triggered by UV light and camptothecin and contained significantly lower caspase activity [71]. Together with the results obtained with Sf-9 cells transformed with P35 and the *Sf caspase-1* RNAi trigger, these results suggest that P-vank-1 augmented BEV-mediated expression in Sf-9 cells by inhibiting apoptosis. Overall, these results indicate that blocking the baculovirus-induced host cell apoptosis response is an effective general strategy that can be used to engineer insect cell lines for improved expression.

Finally, it is conceivable that one could improve recombinant gene expression in insect cells by introducing genes that improve the utilization of nutrients and decrease the production of toxic by-products during cell culture. In particular, Tn-5 cells generate relatively large quantities of lactate and ammonia during culture [72, 73], which likely have adverse effects on cell health, viability, and

recombinant protein expression [74]. Thus, Elias and coworkers [75] transformed Tn-5 cells with the gene for yeast pyruvate carboxylase, which converts pyruvate to oxaloacetate, a TCA cycle intermediate, and channels the products of glycolysis to the TCA cycle, thereby preventing their fermentation to lactate. Clones of the resulting cell line (Tn-5-PYC) grew to higher cell densities, consumed lower amounts of glutamine and asparagine, metabolized a higher percentage of glucose through the TCA cycle, and produced 40–70 % less lactate and 40–50 % less ammonia than the parental Tn-5 cells [73, 75]. However, the quantity of recombinant β-galactosidase produced in the cells with a baculovirus vector was unaffected [75].

## 4 Notes

1. Generally, our experience with transfecting cells with multiple plasmids suggests that (a) it is necessary to screen more clones to identify one that has incorporated every plasmid, and, (b) the larger the number of plasmids involved in the initial transfection, the more clones that need to be screened.
2. In our experience, transformants do not regain susceptibility to the antibiotic used during selection. Hence, it is necessary to use antibiotics with different modes of action when carrying out sequential transformation to introduce multiple genes into lepidopteran insect cells.
3. We typically start with Sf-9 cells when making transformed cell lines. Sf-9 cells are small, round cells with a highly refractile appearance when examined with a phase-contrast microscope. They have a doubling time of approximately 24 h, although growth rates can vary from passage to passage. We typically maintain the parental Sf-9 cell line using an initial seeding density of $0.3–0.5 \times 10^6$ cells/mL and these cells typically achieve a density of $2–4 \times 10^6$ cells after 2 days of growth in 50 mL shake-flask cultures. However, we have found that some stably transformed cell lines, such as SfSWT-3 [22], benefit from slightly higher seeding densities (e.g., $1.5–2.0 \times 10^6$ cells/mL) with respect to their overall appearance, doubling times, and final densities.
4. Lectin blotting is an example of a simple, straightforward technique that provides preliminary information on the ability of transformed cells to perform the desired protein modification (in this case, altered *N*-glycosylation). Lectins are proteins that bind to specific carbohydrate structures and can be used to detect these structures on proteins immobilized on a membrane, much like antibodies are used for immunoblotting. To obtain clean, easily interpretable results with lectin blots, it

is necessary to partially purify the glycoprotein of interest beforehand. Immunoprecipitation of the glycoprotein with an antibody accomplishes this task and provides an internal positive control for ability of the lectin to detect the carbohydrate structure of interest. It is also necessary to include negative controls in which the lectins are preincubated with an excess of the appropriate competing sugar. Finally, an additional control is to pretreat the recombinant glycoprotein with endoglycosidases or exoglycosidases that will remove all or part of the *N*-glycan prior to the lectin binding assay.

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# Part VI

## Baculovirus Development and Production for Use as Insecticides

# Chapter 19

# Introduction to the Use of Baculoviruses as Biological Insecticides

**Holly J.R. Popham, Tyasning Nusawardani, and Bryony C. Bonning**

## Abstract

Baculoviruses are widely used both as protein expression vectors and as insect pest control agents. This section provides an overview of the baculovirus life cycle and use of baculoviruses as insecticidal agents. This chapter includes discussion of the pros and cons for use of baculoviruses as insecticides, and progress made in genetic enhancement of baculoviruses for improved insecticidal efficacy. These viruses are used extensively for control of insect pests in a diverse range of agricultural and forest habitats.

**Key words** Baculovirus insecticides, Recombinant baculovirus, Insect pest management

## 1 Baculovirus Life Cycle

Baculoviruses have been isolated from more than 400 insect species [1]. Most known baculoviruses are nucleopolyhedroviruses (NPVs; genus *Alphabaculovirus*) or granuloviruses (GVs; genus *Betabaculovirus*) mainly within Lepidoptera (butterflies and moths) [2]. They are identified by occlusion body (OB) morphology with single (GV) and multiple (NPV) virions (occlusion-derived virus, ODV) occluded in granules and polyhedra, respectively. Infection occurs following ingestion of virus-contaminated foliage by a susceptible host insect. On ingestion of occlusion bodies (e.g., polyhedra), the polyhedrin matrix dissolves in the alkaline midgut and occlusion-derived virus (ODV) is released (Fig. 1). The ODV pass through the peritrophic membrane and enter the columnar cells of the midgut epithelium [3–5]. Virus replication takes place in the cell nucleus and early replication results in production of a second viral phenotype, the budded virus (BV) (Fig. 1). Budded virus circulates in the hemolymph and initiates secondary infection throughout the host [6]. The midgut, hemocytes, tracheal matrix, and fat body cells are the most readily infected tissues in susceptible hosts [7].

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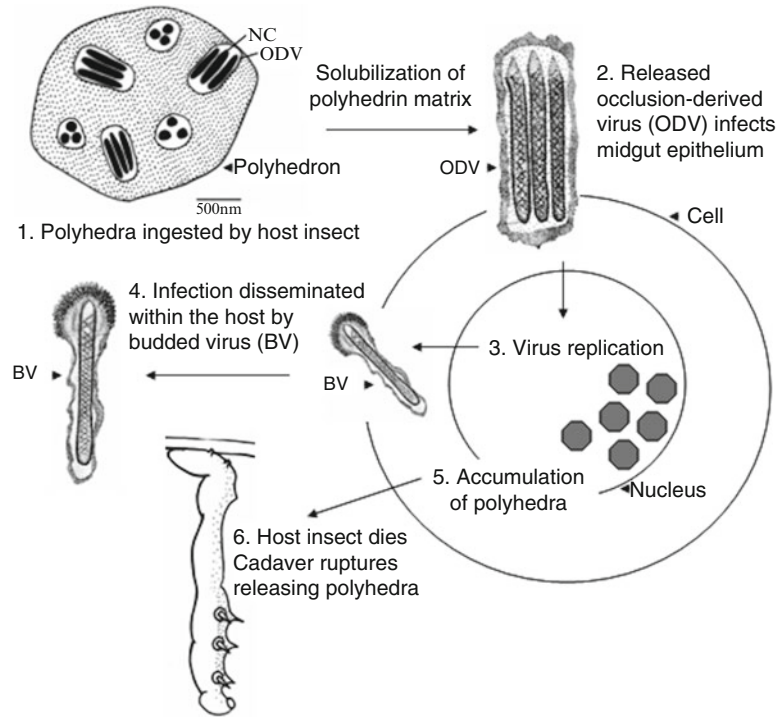


**Fig. 1** Life cycle of a multiple nucleopolyhedrovirus (MNPV). MNPV have multiple nucleocapsids (NC) within occlusion-derived virus (ODV). The two baculovirus phenotypes, ODV and budded virus (BV), are illustrated showing the NC within each virion type

A transition occurs during the late phase of infection from BV production to ODV and polyhedra production. Polyhedra accumulation within the nuclei of epidermal and fat body tissues results in pale coloration of larvae at very late infection stages. At advanced stages of disease, polyhedra are released into the hemolymph. Larvae appear swollen and become lethargic, and typically exhibit negative geotropism prior to death, which may facilitate polyhedra dispersal. Upon death the larval cuticle ruptures easily, thereby releasing polyhedra into the environment. Depending on the virus-host combination and environmental conditions, a virus may take from 5 days to 3 weeks to kill the host.

## 2 Commercial Use of Baculovirus Insecticides

Both alphabaculoviruses and betabaculoviruses have been used extensively for control of insect pests in a diverse range of uses, including row crops, orchards, stored products, and forests [1, 8]. As many as 50 formulations of baculoviruses have been registered worldwide and used to varying degrees of success [9]. Commercial

production has been carried out only in vivo either by harvesting infected larvae from the field or by infecting larvae reared on artificial diet [10], though work continues to improve cell lines for virus biopesticide production [11]. Formulation and application of baculoviruses are described elsewhere for apple orchards [12], cotton [13], forest [14], soybean [15], and general pest control [16].

One of the most successful examples of baculovirus use is the application of *Anticarsia gemmatalis* MNPV for velvet bean caterpillar management in soybeans in Brazil, where at one time it was applied to more than one million hectares annually [17]. However, the use of AgMNPV declined after the implementation of no-till agricultural systems and the occurrence of soybean rust in Brazil which caused growers to use herbicide mixed with broad-spectrum insecticides as well as fungicides [10]. These practices changed the pest complex as beneficial insects were lost and the soybean system equilibrium was disturbed. At present AgMNPV is used on approximately 300,000 ha/year in Brazil [10].

Heliothine NPVs have been used in both agricultural and horticultural crops for control of corn earworm and old world bollworm in the USA, China, India, and Australia [18, 19]. *Helicoverpa armigera* NPV has been successfully used for the control of the cotton bollworm in sorghum, cotton, and horticulture in Australia [20]. Seven baculoviruses and granuloviruses have been registered as commercial insecticides and used in China, including HearNPV [21].

A diverse group of baculoviruses has been applied in orchard and forest ecosystems. Baculoviruses, either naturally occurring or applied, tend to be more stable in forest ecosystems than agricultural systems because they remain in the environment for long periods [10]. One of the earliest examples of the use of a baculovirus insecticide was application of a baculovirus in 1892 for management of *Lymantria monacha* in German pine forests [22]. *Cydia pomonella* GV has been successfully applied in orchards to control codling moth in North America, Europe, Argentina, Australia, New Zealand, and South Africa [23]. *Neodiprion abietis* NPV, a gammabaculovirus, has been used for control of balsam fir sawflies in Canadian fir forests [24]. One of the most successful BVs used in the USA for pest control is Gypcheck (LdMNPV), an NPV for the control of gypsy moth, *Lymantria dispar* [25].

## 3 Pros and Cons of Baculovirus Insecticide Use

Baculoviruses are attractive biopesticides because they are environmentally safe, insect-specific, pose no risk to human health, and have excellent compatibility with sustainable farming practices [17, 18]. They are also harmless to nontarget organisms including beneficial predators and parasitoids as well as vertebrates and plants

[26–28]. They persist for many years in environments such as the soil where they are protected from light, thereby indicating they have epizootic potential as well as inundative control use [1, 29].

While baculoviruses are widely used, their current use falls far short of their potential as effective biocontrol agents due to several limitations. These include problems with long term storage, high cost of production relative to classical chemical pesticides due to the need of a living system for production, reduced persistence in the field when exposed to ultraviolet light, and relatively slow speed of kill of the targeted pest [18, 30]. In addition, a new concern in orchards is the recently identified resistance of codling moth to CpGV [31]. The narrow host range of baculoviruses is restrictive for management of multiple pest species in a cropping system. The stability of baculoviruses can also be impacted by temperature, pH, and moisture, as well as plant metabolites and other environmental factors [19, 32]. Once a baculovirus is applied, the infected larvae continue to feed for several days until their death and may cause significant damage. Growers expect baculoviruses to act similar to fast-acting classical chemical insecticides. Some cropping systems, e.g., pine forests, can tolerate the damage that occurs during this period without economic loss. The cost of such damage to orchard and field crops, however, is too great and limits the commercial use of baculovirus insecticides in these settings. For best control, growers must carefully monitor crops for pest insects to time virus applications against the most susceptible larval instars [10].

## 4 Genetic Enhancement of Baculovirus Insecticides

Many recombinant baculoviruses have been constructed with the specific goal of enhancing the insecticidal properties of the virus [30, 33]. Most of these viruses have been constructed to reduce the time taken by the virus to kill the host insect, although genetic manipulation of baculovirus host range [34] and improvement of virus stability [35, 36] have also received some attention. On occasion, recombinant baculoviruses constructed for protein expression purposes have been shown to have interesting effects on insects. For example, overexpression of the insect hormone, prothoracicotrophic hormone, decreased the pathogenicity of the baculovirus [37]; a baculovirus expressing a maize protein that disrupts the inner mitochondrial membrane was insecticidal [38]; and a catalytically inactive insect enzyme, juvenile hormone esterase, was toxic to baculovirus infected larvae [39] or a mutated juvenile hormone esterase caused a reduction in larval mass and feeding damage [40].

The classic methods developed for constructing recombinant baculoviruses for protein expression purposes [41–43] have been

used for production of recombinant baculovirus insecticides. Clever new methods have improved the production of recombinant baculoviruses largely through the construction of bacmids [5, 44]. The main difference in the approaches to generate recombinant proteins and bioinsecticides is the requirement for baculovirus insecticides to express the polyhedrin gene. The polyhedrin matrix that embeds the ODV confers protection against desiccation and freezing [45]. Polyhedrin is not needed for the in vitro infection of insect cells. Therefore, the foreign gene is commonly inserted into the baculovirus genome in place of the polyhedrin gene to express a recombinant protein under control of the strong polyhedrin promoter.

Three approaches have been used for producing genetically enhanced baculovirus insecticides with reduced kill time that can be used either alone or in combination: (1) insertion of a gene encoding an insect-specific toxin, hormone or enzyme, (2) deletion of a baculovirus gene and/or (3) incorporation of a toxin into the occlusion body. Only a few key examples will be described here. For comprehensive information on other recombinant baculovirus insecticides, the reader is referred to Harrison and Hoover [3], Kamita et al. [30] and Inceoglu et al. [33].

On replication of the baculovirus within the cells of the infected host, the product of the foreign gene (toxin, hormone or enzyme) is expressed along with the baculovirus proteins. Expression of these toxic agents has a deleterious impact on the host insect. Hence, the baculovirus serves as a delivery system for the toxic agent and death results from expression of the toxin rather than from the baculovirus infection. The insect-specificity of the expressed toxic agent and the limited host range of the baculovirus both contribute to the safety of recombinant baculovirus insecticides [10, 30].

### 4.1 Toxins

Among the most successful examples of genetic enhancement to reduce the time to kill are several baculoviruses that express neurotoxins derived from various venomous animals. These include the toxins tox34.4 derived from the straw-itch mite [46, 47], AaIT and LqhIT2 derived from scorpions [48–50], μ-Aga-IV derived from a spider [51], and As II and Sh I derived from sea anemones [52]. Recombinant viruses expressing these toxins were from 50 to 60 % faster than the respective wild type virus. A baculovirus that expresses a basement membrane-degrading protease had similar efficacy [53, 54]. The genetic manipulation and improvement in speed of kill varies with promoter, the parent virus, host insect strain and stage, virus dose, and the infection method used. Hence, the performance of a baculovirus expressing a given toxin can be enhanced by altering the timing or level of expression (by using different promoters to drive expression), or by employing a different parent virus to target specific pest species [30]. The most

important parameter for pest management, however, is reducing feeding damage, rather than speed of kill. These two parameters may not be directly related, e.g., neurotoxins that paralyze larvae may show a greater reduction in feeding damage than expected from their speed of kill [55].

### 4.2 Ecdysteroid UDP-Glucosyltransferase

The most successful example of the use of gene deletion to enhance baculovirus pathogenicity involves deletion of the baculovirus ecdysteroid UDP-glucosyltransferase gene (*egt*). Deletion of *egt* increases the speed with which the virus kills the host insect. The enzyme Egt catalyzes the conjugation of sugar molecules to ecdysteroids, thereby preventing ecdysteroid entry into cells and effectively inactivating these hormones [56]. The baculovirus expressed Egt prevents molting and pupation of the host insect and provides a selective advantage to the virus in allowing for production of more progeny virus. Deletion or inactivation of the egt gene often reduces survival time of NPV-infected larvae to a significant extent [57–59].

### 4.3 Bt

Another effective strategy for increasing the speed of kill has been incorporation of a Bt toxin into the polyhedrin matrix. Chang et al. [60] expressed both the native polyhedrin protein and a fusion protein consisting of polyhedrin fused to the Cry1Ac toxin and GFP. This fusion protein became incorporated into the polyhedrin matrix. Bt toxins such as Cry1Ac bind to receptors in midgut epithelial cells and cause osmotic cell lysis. The presence of the Bt toxin in the polyhedrin matrix reduced the $LC_{50}$ of the baculovirus approximately 100-fold, and the speed of kill was improved 63 % compared to the wild type virus [60]. Shim et al. [61] constructed a virus expressing both Cry1-5 and the AaIT neurotoxin in *Autographa californica* MNPV that also killed larvae faster than wild-type virus.

### 4.4 Field Testing of Recombinant Viruses

Recombinant baculovirus insecticides have been tested in the field in the USA and in the UK. Trials in the USA demonstrated that recombinant baculoviruses are competitive with fast-acting chemical insecticides under field conditions in terms of preventing economic loss [62, 63]. Trials of recombinant baculovirus insecticides have been conducted recently in China [21, 64], where practical use on a wide scale may be seen. Overall, recombinant baculovirus insecticides have potential for widespread use for insect pest management, particularly in developing countries and for management of pests that are resistant to classical chemical insecticides.

## 5 Future Use

There have been many attempts to overcome the inherent drawbacks of baculoviruses and to develop them as valuable biocontrol agents. The production of recombinant viruses with

gene deletion/insertion has improved the efficacy of several baculoviruses [33]. Nonetheless, a strong negative social perception of genetically modified organisms has had important consequences for developing baculoviruses as biocontrol agents [9]. To date, no recombinant viruses have been registered for agricultural use. In their recent position paper, the Ecological Society of America recommended that genetically engineered organisms be designed to reduce environmental risk, that more extensive studies of environmental benefits and risks be conducted, and that environmental release of these organisms should be prevented if scientific knowledge about possible risks remains inadequate [65]. They identified specific risks associated with recombinant viruses including possible gene flow between recombinant and wild-type viruses in the field and unknown negative effects on nontarget organisms [65]. Before field trials of recombinant virus are conducted on a larger scale, careful thought must also be given to the persistence, competitive fitness, and ecological impact on target and nontarget hosts as well as naturally occurring pathogens [66–68]. Despite reservations over the use of genetically engineered organisms, there remains an urgent need for the development of environmentally safe biopesticides for global use [10]. The use of these recombinants will likely increase in the coming years as the application of chemical pesticides becomes less palatable to the public.

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# Chapter 20

# Baculovirus Insecticide Production in Insect Larvae

**Nikolai van Beek and David C. Davis**

## Abstract

Baculovirus-based insecticides are currently being used worldwide, and new products are in development in many countries. The most dramatic examples of successful baculovirus insecticides are found in soybean in Brazil and cotton in China. Production of baculoviruses is generally done in larvae of a convenient host species, and the level of sophistication varies tremendously between field-collection of infected insects at the one extreme and automated mass manufacturing at the other. Currently, only products with wild type baculoviruses as active ingredients are commercially available. Baculoviruses encoding insecticidal proteins are considered attractive, especially for crops with little tolerance to feeding damage, where speed-of-kill is an important characteristic. Successful field tests with such recombinant baculoviruses have been done in the past, and more tests are ongoing. However, low-cost production of recombinant baculovirus in larvae poses specific problems, due to the short survival time of the production host.

In this chapter, benchtop-scale production of two typical baculoviruses is described. First, we describe the production of wild type *Helicoverpa zea* nucleopolyhedrovirus in bollworm (*H. zea*) larvae. *H. zea* larvae are very aggressive and need to be reared in isolation from each other. Second, we describe the production of a recombinant *Autographa californica* multiple nucleopolyhedrovirus in the non-cannibalistic cabbage looper, *Trichoplusia ni*. The recombinant baculovirus encodes the insect-specific scorpion toxin LqhIT2. The tetracycline transactivator system enables the production of wild-type quantity and quality product while toxin expression is repressed since normal toxin production would result in premature death of the production host that would limit progeny virus production.

**Key words** *Helicoverpa zea* nucleopolyhedrovirus, Corn earworm, *Trichoplusia ni*, Cabbage looper, Baculovirus production, Recombinant *Autographa californica* multiple nucleopolyhedrovirus, Tetracycline transactivator, LqhIT2

## 1 Introduction

Baculovirus-based insecticides are being used to control a variety of lepidopteran pests in many countries [1]. Products are on the market in Brazil (soybean looper), Switzerland and Germany (codling moth), France (codling moth and other Lepidoptera), the USA (bollworm), Canada (codling moth), China (various Lepidoptera), Peru (potato tuber moth), Russia (various Lepidoptera), India (old world bollworm, armyworms), and South Africa (false codling moth). In Japan (leafrollers), Vietnam and Thailand (diamondback

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moth and beet armyworm) baculovirus-based insecticides have been registered or are in development. The crops and target pest insects vary, although almost all commercial products are directed against Lepidoptera.

The largest virus producers are found in China and Brazil. In China, one hundred tons of bioinsecticide based on the *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV) are produced annually for use on cotton by several commercial companies (e.g., Trend Biotechnological Lmt. Co., Ezhou City, Hubei Province; Tianmen Bioinsecticide Factory, Jianghu, Tianmen City, Hubei Province). A number of other baculoviruses are produced on a smaller scale (*Spodoptera litura* NPV, *Autographa californica* MNPV, *Plutella xylostella* granulovirus (GV), and *Gynaephora ruoergensis* NPV). In Brazil alone, approximately 2 million hectares (Ha) of soybeans were treated with *Anticarsia gemmatalis* MNPV (AgMNPV) during the 2003/2004 season [2] with product manufactured by a number of companies, one of which (Coodetec) had the capacity to manufacture 1.4 million Ha treatments per year. Due to the implementation of no-till agriculture with the concomitant application of herbicides and broad-spectrum chemical insecticides, the soybean treatment with AgMNPV decreased to approximately 300,000 Ha by the 2010/2011 season. The product is also sold in neighboring countries (i.e., Paraguay and Bolivia). In India, there are several companies involved in baculovirus production, mostly HaNPV. Among the most important commercial producers are Ajay Biotech (Pune), Basarass Biocontrol (Chennai), Pest control India (Bangalore), SOM Phytopharma (Hyderabad), and Sun Agrochem (Indore). Large-scale commercial baculovirus manufacturing is in development by Pest Control India as current manufacturing cannot satisfy demand. In Europe, as well as in the USA, the codling moth is a serious pest in orchards and is treated with *Cydia pomonella* GV, which is produced in several western European countries, Russia, and Canada. The US Department of Agriculture has produced baculovirus against forest insect pests, but commercially produced virus against the cotton pests *H. zea* and *Heliothis virescens* (Certis USA, Columbia, MD) is mostly exported to Australia as it is also very active against *H. armigera*. Except for the producer BioTepp, baculovirus production in Canada is mostly in the hands of government agencies, which produce registered virus products against four forest pests: Douglas fir tussock moth, gypsy moth, white marked tussock moth, and redheaded pine sawfly [3]. In Africa there are several examples of promising baculovirus programs. Tanzania has waived registration requirements for *S. exempta* NPV, and efforts to produce this virus are ongoing in collaboration with a Brazilian producer (EMBRAPA). Dudutech in Kenya is developing a virus against *P. xylostella* for use on cole crops. In South Africa, River Bioscience has commercialized a GV against *Cryptophlebia leucotreta* on citrus. CIP

(International Potato Center) in Peru produces a GV against *Phthorimaea operculella* on stored potatoes in Tunisia and Egypt. Thailand and Vietnam have active programs to promote the use of baculoviruses, and governmental agencies have produced limited quantities of insecticide, especially *S. exigua* NPV against the beet armyworm on grapes, soybeans, onions, and cabbage.

The level of technical sophistication of these manufacturing processes varies widely, from cottage industry type operations, often found in regions where labor is inexpensive, to highly automated production processes. In some cases the farmers and their families collect diseased insects in the field which are then prepared for use as bioinsecticide, sometimes in mixtures with *Bacillus thuringiensis* or synthetic insecticides. At this end of the spectrum of manufacturing methods the quality and consistency of the product is usually very low. At the other end are sophisticated commercial producers with quality assurance and control practices built in at all levels of the process.

Since baculovirus-induced cessation of feeding, especially in the case of vegetable pests, is often too slow to prevent economic damage, attempts have been made to speed up the response. It was quickly recognized that the baculovirus expression vector system could be used to deliver insecticidal genes into the insect (*see* Chapter 19). Eventually, recombinant viruses expressing scorpion and spider toxins showed such promise that agrochemical companies initiated R&D programs. The first was Sandoz Crop Protection in the late 1980s, followed by Zeneca, DuPont, and American Cyanamid starting in the early 1990s. The latter two companies developed baculoviruses encoding a scorpion toxin, and carried the development of baculovirus-based insecticides close to commercialization [4]. While American Cyanamid focused on cell culture production methods, Dupont researched the production of recombinant baculoviruses in larvae. To that end Dupont built a pilot facility in Newark, Delaware, with an annual capacity of approximately 40,000 acre-treatments of recombinant *H. zea* NPV (HzNPV).

We focus in this chapter on small-scale virus production, which provides sufficient virus for bioassays and small-scale field testing [5]. The method used to produce wild type virus in larvae cannot be used for the production of recombinant virus. For instance, any recombinant virus that is effective in shortening the time-to-kill limits the multiplication potential of the virus and consequently the yield of progeny viral occlusion bodies (OBs). In the case of HzNPV carrying the gene of the insecticidal scorpion toxin, *Leiurus quinquestriatus hebraeus* LqhIT2, time-to-kill is reduced by 70 % [6] and virus yield is reduced by 90 % (Davis and van Beek, unpublished results) compared to wild type-infected neonate and fifth instar *H. zea* larvae, respectively.

We describe methods for producing wild type HzNPV and recombinant *Autographa californica* MNPV (AcMNPV) in individually reared *H. zea* and group-reared *Trichoplusia ni* larvae, respectively. The recombinant AcMNPV encodes the insecticidal LqhIT2 under the control of a promoter whose activity is regulated by the tetracycline transactivator system.

## 2 Materials

### 2.1 Rearing of H. zea Larvae and Determination of Larval Instar

1. Insects: *H. zea* can be purchased from Benzon Research (Carlisle, PA, www.benzonresearch.com) and Bio-Serv (Frenchtown, NJ, www.bio-serv.com).
2. Insect rearing trays: approximate dimensions of the wells in the trays, 1″ by 1″ by 0.875″ (length by width by depth). The rearing trays can be purchased either empty or already filled with the insect diet (e.g., Bio-Serv).
3. Insect diet, e.g., General Purpose Lepidoptera Diet (Bio-Serv, product #F9772).
4. Sterilized corn cob grits (e.g., Bio-Serv).
5. Hand-operated plastic inoculator (often called a bazooka) and mixing bottle or hopper (Bio-Serv, products #9050 and 9146, respectively).
6. Transfer forceps (e.g., BioQuip Products, Rancho Dominguez, CA, product #4750, www.bioquip.com).
7. Perforated Mylar—27HT1/PET translucent polyester film (Oliver Products Inc., Grand Rapids, MI).
8. An iron, for heat sealing the Mylar lidding onto the tray.

### 2.2 Occlusion Body Concentration Determination by Hemacytometer Count

1. Hemacytometer plus 0.4-mm coverslips: e.g., Bright Line Counting Chamber (Hausser Scientific, Horsham, PA, product #3100).
2. Tween 20 (Sigma-Aldrich, St. Louis, MO): 50 % solution in water.

### 2.3 Larval Harvesting and Downstream Processing

1. 100 Mesh plastic screen (e.g., www.miami-aquaculture.com, Boynton Beach, FL) or four layers of cheesecloth (e.g., www.thegadgetsource.com, Ashville, NC).

### 2.4 Production of Recombinant AcMNPV in T. ni Larvae

1. Doxycycline: 5 mg doxycycline (Sigma-Aldrich) per mL inoculum suspension, prepared fresh before inoculation.

## 3 Methods

### 3.1 Production of Wild Type HzNPV in H. zea Larvae

The corn earworm, when reared at its optimal temperature (29 °C) on a suitable diet [7, 8], develops from egg to pupa in about 16 days. The insect spends approximately 2 days in the egg stage, followed by 12 days in the larval stage as the larva progresses through five larval instars (Fig. 1). At the end of the fifth instar the insect enters a pre-pupal stage for approximately 2 days. Metamorphosis takes place in the pupal stage, and after about 8 days the adult insect emerges. Unlike *T. ni*, *H. zea* larvae are extremely cannibalistic [9], especially during the fifth instar, and require isolation in individual rearing units (Fig. 1). For the purpose of baculovirus production, the late fourth instar larval stage is ideal for oral inoculation with baculovirus, which will result in recovery of about $10^{10}$ OBs per larva.

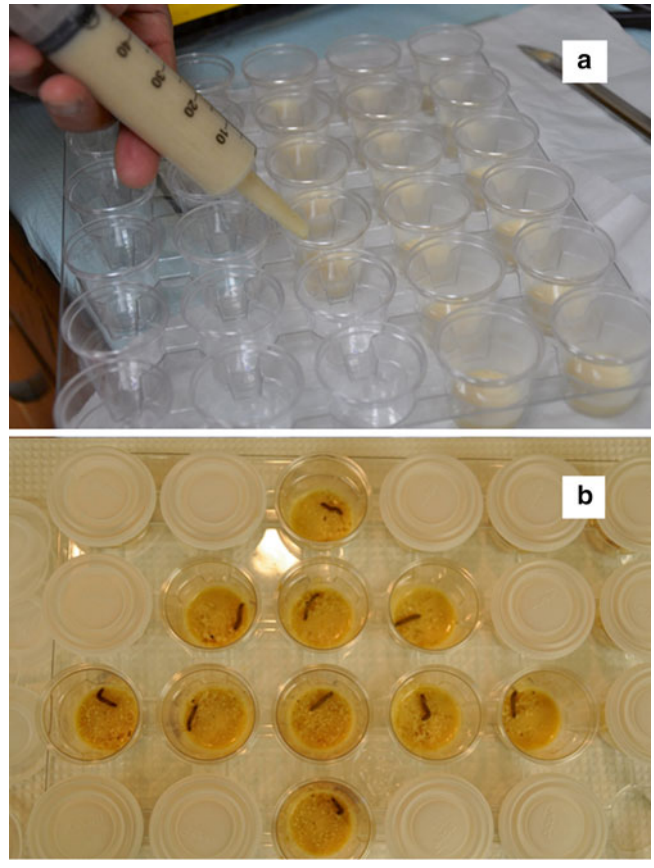


**Fig. 1** Early fifth instar *H. zea* larvae in a rearing tray from which the Mylar lid is partly removed. Note the debris, insect excrements (frass) and corn cob grits, covering the diet

### 3.2 Rearing of H. zea Larvae and Determination of Larval Instar

This protocol describes a labor-intensive method in which larvae are grown separately until they are ready for inoculation and then transferred one-by-one into a new tray containing virus-treated diet. This method is reasonably fail-proof and should result in good quality progeny virus. The most critical parameter for success is whether the OB concentration determination is correct (*see* **Note 1**). After becoming more familiar with the insect's behavior and appearance, or when very large amounts of virus are needed, the researcher may choose to simplify the method given below (*see* **Note 2**).

1. Determine the quantity of *H. zea* eggs. Ten eggs weigh approximately 1 mg.
2. Based on roughly five eggs and 150 mg corn cob grits per well, calculate and weigh the required amount of grits.
3. Add 1 mL water for each portion of 15 g grits and mix until clumps have disappeared.
4. Mix *H. zea* eggs and moistened grits (3.4 mg eggs/g) and incubate 18–24 h at 29 °C and 60 % relative humidity (RH).
5. If prepared insect diet is purchased ready for use in multicellular trays (e.g., Bio-Serv), then proceed to **step 9**. The diet may also be prepared from a dry bulk pre-mix or from individual dietary components, which may be purchased from any of several vendors. Follow the manufacturer's instructions for preparation of the specific diet; alternatively, *see* elsewhere in this volume for a procedure to prepare a General Purpose Lepidoptera diet suitable for *H. zea* propagation (e.g., *see* Chapter 13, Subheading 3.2).
6. After preparation, place the liquid diet in a water bath at approximately 44 °C to prevent it from solidifying prior to dispensing. Spread out empty plastic trays on a table.
7. Modify a soft plastic wash bottle (500 mL) by removing the draw tube and cutting off the elbowed dispensing neck, creating a straight stem. Rinse the bottle well with warm water, and fill with the liquid diet. Invert the wash bottle over the multicellular rearing tray and dispense diet into the wells of the trays. Fill the wells to approximately one-third with diet. Let the trays stand for about 15 min to allow the diet to solidify and cool to room temperature. Any drops of diet that have formed outside of the wells can be easily wiped away once the diet has set.
8. Use a hand-operated plastic inoculator to dispense 150 mg of the mixture of pre-incubated corn cob grits and eggs into each well. The trays are purposely seeded with an average of five eggs to compensate for eggs that do not hatch and to ensure that all wells contain at least one larva. *H. zea* larvae are

cannibalistic and the density will decrease to one larva per well by the fifth instar.

9. Overlay the tray with perforated Mylar film, smooth side up, and quickly seal using an iron at a low setting. Overheating the tray with the iron will cause the tray to soften and lose its shape.
10. Incubate the trays at 29 °C and 60 % RH to prevent the diet from drying out.
11. After 4–6 days' incubation, begin to monitor larval development. The larvae are ready for inoculation when most of them are in the fourth instar and a small percentage (less than 10 %) have reached fifth instar (*see* **Note 3**).

### *3.3 Occlusion Body Concentration Determination by Hemacytometer Count*

1. Prepare a homogeneous stock suspension of HzNPV OBs by sonication for 20 s at medium setting (avoid formation of a large number of air bubbles in suspension). Add 1/100 volume of 50 % Tween 20, and mix well.
2. Prepare independently six serial dilutions in glass tubes using an automatic pipettor. When starting with a concentrated stock suspension, we recommend making a 1:100 dilution first, by adding 100 μL stock suspension to 9.9 mL water. Mix thoroughly and then prepare five serial tenfold dilutions (1 mL virus suspension in 9 mL water). Mix well before each subsequent dilution.
3. Before use, clean the hemacytometer and the coverslip with 70 % alcohol and blot dry with tissue. Place the coverslip on the hemacytometer supports.
4. To become familiar with the size and appearance of HzNPV OBs (*see* **Note 4**), load the hemacytometer with about 10 μL of concentrated OB suspension. Let the hemacytometer stand for 15 min to allow the OBs to settle at the bottom of the chamber. Observe the OBs under a microscope, using phase contrast at 400× magnification (*see* **Note 5**).
5. After vortexing the highest dilution of one series, withdraw 10 μL and load one chamber of the hemacytometer by placing the pipet tip into the notch and gently dispensing the aliquot into the chamber. Fill the other chamber in the same manner with the second-highest dilution. Place the hemacytometer under a Petri dish lid on a moist paper towel and let stand for 15 min.
6. Using microfocus to highlight the unique shape and light refraction properties of the OBs, and to count all OBs throughout the 0.1 mm depth below the coverslip, the four corner groups of squares and the central group are counted, for a total of 80 small squares (*see* **Note 6**).

7. The total number of OBs in the five groups of squares for both grids is counted. Select a member of the dilution series with counts between 20 and 200 OBs. Count the OBs in the equivalent dilution of the remaining five dilution series.
8. From the six counts, eliminate the highest and lowest values and determine the average of the remaining four. A reliable count should have a coefficient of variation below 10 %. Make new counts if this is not the case. If there seems to be a systematic difference between the dilution series, then prepare new dilutions.
9. Calculate the OB concentration of the stock suspension as follows: The OB concentration (OBs/mL) equals the average count multiplied by the dilution factor and divided by the volume counted (mL). The volume of the five groups of squares from **Step 7** is **5 x 10** mL (each group of squares has a grid of 0.1 x 0.1 cm, and a chamber depth of 0.01 cm, which is a volume of **10** mL).

### 3.4 Inoculation

1. To obtain $10^{13}$ HzSNPV OBs, prepare rearing trays containing 1000–1100 wells filled with roughly 4 mL insect diet per well. Trays prepared earlier and refrigerated may be used, after warming to room temperature (*see* **Note 7**).
2. Prepare a 60-mL viral suspension of $2 \times 10^4$ OBs/mL in dechlorinated water. A food-coloring dye may be added as a convenient means to mark trays that have been treated.
3. Pipet 50 μL OB suspension onto the diet surface of each well. Allow 10–15 min for the inoculum to dry before introducing larvae.
4. Seed the treated trays with late fourth instar corn earworm larvae, loosely covering the tray with perforated Mylar film to prevent escape. Once a tray is completely seeded, seal the Mylar with a warm iron. Repeat this step until all trays are seeded.
5. Incubate the treated larvae at 28 °C, 30–50 % RH.
6. Monitor for mortality. Harvest when about 90 % of the larvae are dead. The harvest time should be around 120 h after inoculation.

### 3.5 Larval Harvesting and Downstream Processing of HzNPV OBs

The larval cadavers will be very fragile and difficult to harvest manually. Use care when handling the trays so as not to expose the cadavers to any forces that could rupture their integument. Vacuum extraction from the tray into a chilled collection container is recommended.

1. Construct a vacuum harvester as follows. Take a glass Erlenmeyer flask with a side port and close the top with a rubber stopper penetrated by a glass tube (e.g., the solid part of a

glass transfer pipet). Connect the side port via vacuum tubing to a vacuum source, and then insert a transfer pipet into one end of a piece of tubing (e.g., Tygon) with the other end over the glass tube sticking out of the rubber stopper.

2. Place the Erlenmeyer flask on ice, turn on the vacuum, and aspirate the larval cadavers from the diet into the flask with the transfer pipet.
3. Combine one part larval cadavers and four parts ice-cold water in a blender. Blend at medium setting for 1–2 min or until all insect material is well homogenized.
4. Pour the crude homogenate through a 100 Mesh screen (or four layers of cheesecloth) to remove gross particulates. If the downstream material needs to pass through a spray nozzle, then a final filtering through 100 Mesh is recommended at this step. Some agitation may be required to enable the material to pass through the 100 Mesh screen. Keep the homogenate chilled during this process to avoid excess bacterial growth.
5. The filtered homogenate can now be centrifuged to concentrate the baculovirus into a paste. Aliquot approximately 400 mL of the homogenate into 500-mL centrifuge bottles. Centrifuge at $12{,}000 \times g$ at 4 °C for 20 min, and then immediately remove the centrifuge bottles and decant the supernatant.
6. Wash the pellet twice by resuspending it in chilled water. Repeat the centrifugation described in **step 5**.
7. After completing the washing process, the pellet ("paste") is collected into a chilled container (stainless steel bowl or glass beaker) and homogenized with a spatula. If necessary, the homogenized paste may be transferred at this stage to a storage container where it can be held at 4 °C for a few days or at −20 °C for longer periods. (However, we prefer to mix the paste with 2 volumes of 50 % glycerol before storage at −20 °C (*see* **Note 8**)).
8. The OB concentration in the paste may be determined by collecting three sub-samples from the homogenized paste and counting the number of OBs present. If the paste sub-samples cannot be quantified immediately, then they may be stored for up to 7 days at 4 °C.
9. The OBs in the pellet should be very concentrated, and virus suspensions for bioassays or field experiments may be prepared by dilution in water. If the virus is to be applied in dry form (e.g., after spray-drying), then note that the presence of glycerol (*see* **Note 8**) has a detrimental effect on the physical properties of the final product and its use should be avoided. The

glycerol can be diluted to a negligible concentration by alternating centrifugation and resuspension of the pellet in water.

### 3.6 Production of Recombinant AcMNPV in T. ni Larvae

The typical effect of recombinant baculovirus insecticide—a reduced response time compared to wild-type virus [10]—is the main cause of decreased yields in larvae infected with recombinant virus. The reduction in yield may be up to 90 %, thereby preventing economical virus production. The problem may not be limited to the number of OBs produced: their size and stability may also be vastly inferior to those of wild-type OBs. In the case of the insecticidal scorpion toxin LqhIT2 [11, 12], these effects are presumably due to cytotoxic activity of the toxin product that reduces cell viability and prevents complete OB maturation (Davis, Joraski, and van Beek, unpublished results). The solution to the yield and OB maturation problem lies in the use of a controllable promoter to express the insecticidal gene. Thus, during virus production, toxin synthesis is suppressed, whereas in the field the toxin is expressed. We describe the production of AcMNPV encoding $LqhIT_2$ [8] under control of the tetracycline transactivator [13]. This method has not been published, but it has been described in a patent [14]. The following protocol should yield from $5 \times 10^{11}$–$10^{12}$ AcNPV-LqhIT2 OBs per 1000 *T. ni* larvae.

1. Rear *T. ni* larvae in 8-oz cups on 20 mL diet, as described in Chapter 13.
2. Determine the OB concentration as described under Subheading 3.3.
3. Prepare a suspension of $3.3 \times 10^4$ recombinant AcMNPV OBs (*see* **Note 9**) in 5 mg/mL doxycycline for surface treatment of the insect diet.
4. Treat the diet surface in 8-oz cups with 300 μL virus/antibiotic suspension. Using a spreading rod, disperse the surface treatment evenly such that the entire diet surface is covered. Allow the surface to dry before placing *T. ni* larvae in the cups.
5. Seed each treated cup with 25 late fourth instar cabbage looper larvae. Determination of the developmental stage of *T. ni* is described in Chapter 13. For those familiar with the method, the head capsule widths of *T. ni* instars are tabulated below (*see* **Note 3**).
6. Incubate the cups at 28 °C, 30–40 % RH for approximately 120 h, and begin monitoring for death at 96 h after inoculation.

### 3.7 Larval Harvesting and Downstream Processing of AcNPV-LqhIT2 OBs

1. Larval harvesting and downstream processing are done as described under Subheading 3.5. The expected yield is between $5 \times 10^8$ and $1 \times 10^9$ recombinant AcNPV OBs per larva (*see* **Note 10**).

## 4 Notes

1. Underestimation of the OB concentration leads to premature death and severely decreased OB yield. Overestimation, on the other hand, results in lower larval mortality and consequently a lower total OB yield.
2. It is possible to simplify this method, especially when the eggs arrive while still attached to the substrate. While *H. zea* larvae will not tolerate a competitor for food and will fight until death when in the fifth instar, they may be reared in groups in 8-oz cups to the fourth instar. The method is the same as for *T. ni*, described in Chapter 13 Subheading 3.3. However, due to the tendency for cannibalism of *H. zea*, it is even more important to limit the number of larvae per cup to 25. Nevertheless, some of the weaker larvae will not survive group rearing, and a proportion of the survivors will have been wounded by their peers as indicated by black spots on their cuticle. Avoiding severely wounded larvae, the rest can be transferred from the 8-oz rearing cup onto the treated diet in trays (one larva per well). Another way to cut labor is described in **Note 7**.
3. The life stage of a larva can be determined most accurately by measuring the width of its head capsule (under a dissecting microscope with a micrometer scale engraved in the ocular lens) or by weighing it (Table 1). Whether the insect is early or late in a particular instar can best be judged by the width of the head capsule in relation to the width of the body. Larvae that have recently molted possess a wide head capsule and a slender body, whereas late in the instar the width of the body exceeds that of the head. It is possible to slow down developmental speed considerably by lowering the incubation temperature for both *H. zea* [15] and *T. ni* larvae [16].
4. HzNPV OBs are uniform in size and roughly 1 μm in diameter. They are subject to Brownian motion until they rest on the bottom of the chamber. Under phase contrast they are surrounded by a "halo" and can best be distinguished from debris by their regular round shape. AcMNPV OBs, on the other hand, vary in size: they are on average larger, with most

**Table 1**
**Weight and head capsule width for each instar of *H. zea* and *T. ni* larvae**

| | ***Helicoverpa zea*** | | | | | ***Trichoplusia ni*** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Stage (instar) | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| Head capsule width (μm) | 290 | 526 | 905 | 1343 | 1900 | 292 | 440 | 700 | 1230 | 1900 |
| Weight, early instar (mg) | 0.3 | 1.2 | 7.9 | 23.3 | 52 | 0.1 | 1.4 | 10.2 | 15.4 | 70.0 |

particles in the range of 1–5 μm. For the larger OBs, one may be able to see under the microscope their irregular, polyhedral shape.

5. If the stock suspension is a very crude preparation, it may be extremely difficult to count OBs. In that case, the recommended alternate protocol is to carry out a pilot experiment with a small number of fourth instar larvae treated according to the production protocol, but with different dilutions of the virus stock (e.g., $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$). The lowest dose that kills almost all insects (less than 5 % pupation) should then be selected for the production run.
6. Rulings on each plateau of the hemacytometer cover 9 $mm^2$. The central square millimeter is divided into 25 groups of 16 small squares, each group separated by triple lines the middle of which is the boundary. OBs touching the upper or left side boundary are tallied, while those touching the lower or right side boundary are not.
7. Another way to cut the labor is to directly inoculate larvae in the same trays they have been reared in. In that case, it is advisable to double the total amount of OBs per well, using four times the volume (200 μL) at half the concentration ($10^4$ OBs/mL), in order to cover the diet surface and to account for OBs being absorbed by insect excrements (frass) and corn cob grits (Fig. 1). If trays covered by heat-sealed Mylar are used, then the most convenient way is to remove the Mylar, inoculate each well separately with a repeating pipettor, and then cover the tray with new or previously used Mylar and heat-seal it in place using an iron.
8. We recommend storage of baculovirus preparations in 50 % glycerol at –20 °C. At this glycerol concentration, the suspension does not freeze and aggregation of OBs (a typical problem for frozen suspensions) is prevented. OB aggregation causes counting of virus to be very difficult and, more importantly, it may strongly affect the outcome of bioassay and field experiments.
9. Baculovirus expression vectors usually do not contain a functional polyhedrin gene. However, in its application as recombinant insecticide, the baculovirus needs the stability provided by the occlusion body, which is composed mostly of polyhedrin.
10. If suppression of toxin expression is incomplete or absent during the infection cycle, large numbers of larvae will be found dead at 96 h after inoculation. Such larvae do not yield OBs in quantity or quality comparable to wild type virus-infected larvae. In fact, the majority of these OBs are very small and they lack environmental stability (van Beek and Joraski, unpublished results).

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# Chapter 21

# Evaluation of the Insecticidal Efficacy of Wild Type and Recombinant Baculoviruses

**Holly J.R. Popham, Mark R. Ellersieck, Huarong Li, and Bryony C. Bonning**

## Abstract

A considerable amount of work has been undertaken to genetically enhance the efficacy of baculovirus insecticides. Following construction of a genetically altered baculovirus, laboratory bioassays are used to quantify various parameters of insecticidal activity such as the median lethal concentration (or dose) required to kill 50 % of infected larvae ($LC_{50}$ or $LD_{50}$), median survival of larvae infected ($ST_{50}$), and feeding damage incurred by infected larvae. In this chapter, protocols are described for a variety of bioassays and the corresponding data analyses for assessment of the insecticidal activity of baculovirus insecticides.

**Key words** Recombinant baculovirus, Insecticidal efficacy, Host range, Droplet feeding, Diet plug bioassay, Feeding damage, Microapplicator, $LC_{50}$, $LD_{50}$, $ST_{50}$

## 1 Introduction

A variety of strategies have been adopted to genetically optimize baculovirus insecticides, e.g., gene deletion and/or the insertion of genes encoding insect specific neurotoxins, hormones, or enzymes. Of the many recombinant baculoviruses, those expressing venom-derived, insect-specific neurotoxins, or a basement membrane degrading protease are among the most effective [1–5]. To assess the virulence and pathogenicity of a recombinant baculovirus, laboratory bioassays must be performed to evaluate various parameters in comparison with the wild type virus. Enhanced insecticidal activity of a recombinant virus may be manifested through (1) a reduction in median lethal dose, $LD_{50}$ (or median lethal concentration, $LC_{50}$), (2) a reduction in median survival time, $ST_{50}$, or (3) a reduction in feeding damage caused by infected larvae to plant material, relative to the appropriate wild type virus. To assess the host range of a wild type or recombinant baculovirus,

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median lethal dose or concentration bioassays are carried out with different host insects.

In this chapter, protocols and notes are provided for bioassays involving infection of larvae by droplet feeding [6], infection of larvae through ingestion of a virus-treated plug of diet, infection of larvae by a microapplicator, and assessment of feeding damage to leaves by infected larvae [1]. Methods are also described for calculating the toxicological parameters $LC_{50}$, $LD_{50}$, and $ST_{50}$ using SAS [7] and Risk Assessment Tools from the Society of Toxicology and Chemistry [8].

## 2 Materials

### 2.1 Baculoviruses

Any recombinant virus and wild type parent virus can be tested (e.g., the wild type clone *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) C6, and the recombinant baculovirus AcMLF9.ScathL that expresses a cathepsin-L-like, basement membrane-degrading protease (ScathL) [1]).

### 2.2 Insects

*Heliothis virescens* (tobacco budworm). Eggs can be purchased from suppliers such as Frontier Agricultural Services (Newark, NJ, USA) or Benzon Research (Carlisle, PA, USA).

### 2.3 Insect Rearing Supplies

1. Incubator.
2. Artificial diet for *H. virescens* is prepared using premixed diet powder (General Purpose Lepidoptera Diet #F9772, Frontier) according to the manufacturer's instructions.
3. Heavy duty blender (1 L or larger).
4. 8 oz. clear plastic tubs with lids (American Plastic, Chattanooga, TN, USA).
5. 1 oz. plastic cups (with caps) (Frontier).
6. Plastic cup tray (30 wells) (Frontier).
7. 60 cc syringe with catheter tip (BD Biosciences, San Jose, CA, NJ, USA).
8. Small paint brushes (size 0 or 00).
9. Featherweight wide tip forceps (Bio-Quip, Rancho Dominguez, CA, USA).

### 2.4 Supplies for Preparation of Polyhedra (Occlusion Bodies)

1. 15 and 50-mL polypropylene or glass tubes.
2. Dounce homogenizer with a pestle for each virus to be purified.
3. Overhead stirrer.
4. 10 % (w/v) sodium azide stock solution stored at 4 °C.
5. 0.1 and 0.5 % (w/v) SDS.

6. 0.5 M NaCl.
7. Hemacytometers.
8. Inverted microscope (Nikon, TMS, Japan).

### 2.5 Bioassay Supplies

1. eLine single channel multidispensing pipette 0.2–10 μL (Sartorius Biohit, Helsinki, Finland).

#### 2.5.1 Droplet Feeding Method

2. Blue food coloring dye.
3. 60 mm Petri dish.
4. 33 % (w/v) sucrose solution.
5. Dissecting scope.

#### 2.5.2 Diet Plug Feed Method

1. 1 oz. diet cups and lids.
2. Blue food coloring dye.
3. 33 % (w/v) sucrose solution.

#### 2.5.3 Microinjection Method

1. Automatic micro-dispensing system PAX 100-2 (microapplicator, Burkard Scientific, Uxbridge, Middlesex, UK).
2. Plastic tuberculin syringes (1 cc) with a 28.5-gauge needles (sharp tip).
3. Plastic tuberculin syringes (1 cc) with 32-gauge needles (blunt-tip, Popper & Sons, Inc., New Hyde Park, NY, USA).

#### 2.5.4 Feeding Damage Method

1. LI-COR 3100 area meter (LI-COR Inc., Lincoln, NE, USA).

#### 2.5.5 Statistical Packages

1. SAS software (SAS Institute Inc., Cary, NC, USA).
2. Risk Assessment Tools from the Society of Toxicology and Chemistry (Pensacola, FL, USA).

## 3 Methods

### 3.1 Insect Rearing

1. *H. virescens* eggs are typically deposited by female moths on cloth. Cut the egg cloth into smaller strips to fit in several plastic containers (8 oz). Place the sealed containers in a ziploc bag to keep larvae from escaping.
2. Place the bags in an incubator at 28 °C with a 14:10 h light–dark (L:D) photoperiod. If the eggs are light yellowish-green, then they will hatch in 2–3 days. As eggs near hatching, they become orange. To delay hatching by no more than a few days, incubate the eggs at 15 °C.
3. Before eggs hatch, prepare diet and pour into 1 oz plastic cups using a large syringe with a catheter tip (Fig. 1a). Place the cups in 30-well plastic trays. One liter of prepared diet will fill

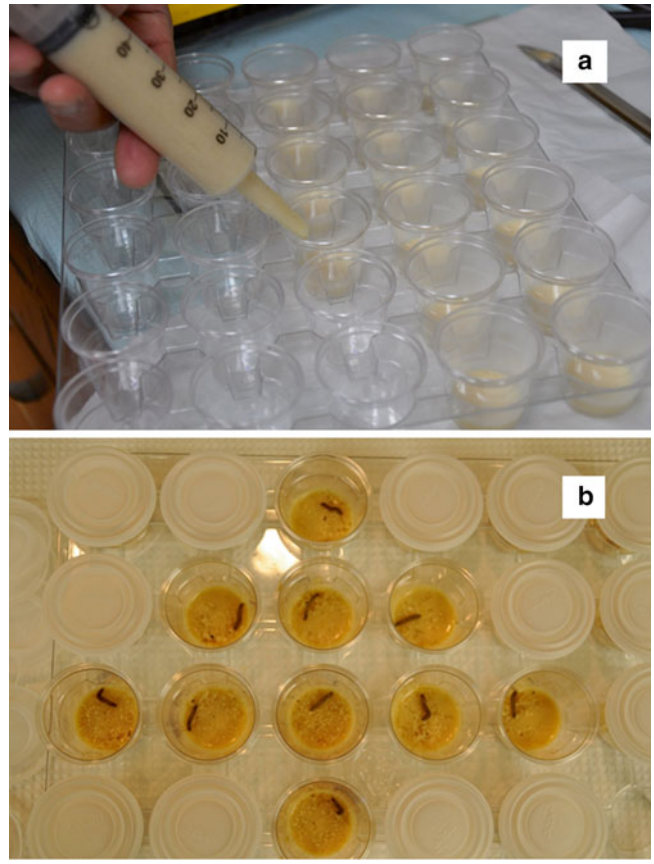


**Fig. 1** Pouring of diet into individual cups. (**a**) After the diet is mixed it is dispensed into cups using a large syringe with catheter tip and (**b**) *H. virescens* larvae are reared individually in cups and grown until the desired instar is reached

approximately six trays of 30 cups each at 5 mL/cup. Diet is wrapped in plastic wrap and stored at 4 °C. Diet should preferably be used within 1 week and certainly before 2 weeks to keep results consistent between assays [9].

4. The brush that is used to transfer neonate larvae must be sterilized by swirling the tip in sodium hydroxide solution (pour a little 10 N NaOH into a small beaker, fill the beaker with about 10 mL of deionized $dH_2O$), then rinsed with ethanol followed by distilled water. Use the brush to transfer one larva to each diet cup. Lightly wetting the brush with water facilitates the transfer of larvae. Many lepidopteran larvae are cannibalistic so it is best to rear them individually or in small groups that can be separated before later instars are reached. Use a featherweight forceps to handle older caterpillars as they will do less damage than rigid forceps.

5. Seal the cups with lids and place the trays in the incubator until the larvae reach fourth instar (Fig. 1b). *H. virescens* larvae go through five or six developmental stages (instars) that are distinguished by the width of the head capsule. As with most lepidopteran larvae, there is variation in the size of the body within an instar, but the head capsule remains the same and only increases in size at the molt from one instar to the next. Immediately before molting, larvae do not feed. The primary indicator of the premolt stage is slippage of the head capsule. When the brown head capsule slips forward and the new, white (untanned) head capsule of the next instar can be seen behind the old head capsule. A white band can be seen between the smaller, previous head capsule and the black band on the thorax. Larvae will molt and shed the old cuticle within several hours of the appearance of the white band. To stop molting while waiting for other larvae to reach the premolt stage, place them at 4 °C. When there are sufficient premolt larvae, return them to 28 °C, and check frequently for newly molted larvae. Alternatively, holding premolt larvae at 15 °C will allow molting and larval growth to continue, but at a slow rate. This cold treatment does not change larval susceptibility to viruses [10].

## 3.2 Preparation of Polyhedra for Bioassay

The virus inoculum used to infect larvae for polyhedra production can be harvested from insect cell culture or virus-killed larvae. Although it is recommended that host insect-derived polyhedra (occlusion bodies, OBs) be used for bioassays [11], polyhedra derived from insect cell culture can also be used. However, it is important that polyhedra are not solely propagated in tissue culture. Genomic modifications often occur in viruses serially passed in cell culture [12, 13]. Budded virus (BV) can also be used for insect infection using microapplicator-assisted injection (*see* Subheading 3.5).

### 3.2.1 Larval Propagation of Polyhedra

1. For amplification of polyhedra, 30–60 larvae are generally needed per virus. However, it is recommended to begin with 60–120 larvae per virus.
2. For polyhedra production, early fifth instar larvae can be infected either by injection of BV into the hemolymph or by oral infection with polyhedra. Fourth instar larvae may need to be infected when studying slower-acting viruses to allow enough time for polyhedra production before larvae pupate.
3. To infect early fifth instar larvae, take fourth instar larvae as they begin to molt to the fifth instar and place them individually in 1-oz. plastic cups without diet at 4 °C. When sufficient larvae are available at the premolt stage, return them to the 28 °C incubator and allow to molt overnight.

4. Transfer the required number of polyhedra ($5 \times 10^4$) on to the diet cube in a small volume (i.e., 1 μL). The larvae will be hungry and will promptly consume the virus-contaminated cube of diet. Alternately, larvae can be infected using the droplet feeding method by feeding newly molted larvae a drop of virus with sucrose and blue food coloring on wax paper as described in the droplet feeding section (*see* Subheading 3.3).
5. When all the larvae have consumed the virus-contaminated diet, provide fresh diet and return larvae to the 28 °C incubator. If larvae are past the premolt stage, then the larvae should still be susceptible to a dose of $5 \times 10^4$ polyhedra, although susceptibility decreases as the larvae age.
6. The infected fifth instar larvae should die 6–8 days post-infection (d pi) for wild type AcMNPV C6 and 3–4 days pi for a fast-acting recombinant baculovirus such as AcMLF9. ScathL. Collect the dead larvae and store in a 50-mL conical tube at −20 °C. Larvae infected with wild type virus become lethargic and pale in coloration prior to death. Do not collect larvae that die within 24 h (when death likely results from handling), if fungus is present or larvae have an unpleasant odor.
7. To harvest polyhedra from the cadavers, place 10–15 cadavers in an autoclaved Dounce tissue homogenizer tube and add 10 mL of 0.1 % SDS. Store the remaining cadavers at −20 °C.
8. Fix the pestle into the overhead stirrer, insert it into the homogenizer tube and then move the homogenizer tube up and down at least ten times to grind the cadavers and release the polyhedra at a suitable spinning speed. Do not grind the tissues excessively to prevent the formation of contaminating fine debris, which will pass through the cheesecloth filter with the polyhedra at the next step.
9. Keep the pestle submerged in the 0.1 % SDS to avoid foaming. If the homogenizer begins to feel warm, then place it in ice for a few minutes to prevent thermal inactivation of polyhedra.
10. Filter the ground larvae through at least five layers of cheesecloth using a funnel.
11. Wrap the cheesecloth around the debris retained by the cheesecloth, and squeeze a few times to release any liquid left in the mass. Do not squeeze it too vigorously otherwise the polyhedra will become contaminated with debris.
12. Transfer the dark brown polyhedra-containing liquid to a Pyrex glass tube (a 50-mL conical tube also works well) and pellet the polyhedra at $900 \times g$ for 10 min at 4 °C. Decant the supernatant into a bleach solution.
13. Wash the gray pellet of polyhedra using 10 mL 0.5 % SDS and then centrifuge again followed by a wash with 10 mL

0.5 M NaCl solution. Repeat until the supernatant becomes clear as necessary.

14. Finally, wash once using sterile $dH_2O$. Resuspend the final pellet in an appropriate volume of sterile $dH_2O$ supplemented with sodium azide at a final concentration of 0.02 % (w/v) as preservative and store at 4 or −20 °C for later use.

### 3.2.2 Cell Culture Propagation of Polyhedra

1. To isolate polyhedra from cultured cell pellets, seed Sf-21 or other susceptible cells in a 75 $cm^2$ flask(s) at ~$5 \times 10^6$ cells per flask.
2. Following attachment of the cells, infect the cells at a multiplicity of infection (MOI) of approximately two and place the flask(s) in a 28 °C incubator.
3. When polyhedra are present in most cells, harvest the cells and place in a 50-mL polypropylene conical tube and store at 4 °C for 2 days. This allows the cells to lyse and release polyhedra.
4. If the polyhedra are needed immediately, then the cell suspension can be centrifuged directly to pellet the polyhedra in a swinging bucket rotor (reduce the volume by 50 % if a fixed angle rotor is used) at 900 × *g* for 10 min at 4 °C. Transfer the supernatant containing BV into a 50-mL polypropylene conical tube and store at 4 °C for later use.
5. Wash the pellet in 10 mL of 0.5 % SDS solution twice (to release the polyhedra from cells), 10 mL of sterile water once, then suspend it in a small volume (i.e., 500 μL) of sterile water and store at 4 or −20 °C.

### 3.2.3 Quantification of Polyhedra

1. Polyhedra are counted by using a hemacytometer and inverted microscope (Fig. 2a). Thoroughly mix the stock of polyhedra by vortexing and dilute with sterile $dH_2O$ in a 1.5-mL centrifuge tube. After appropriate dilution, carefully load the diluted suspension on both sides of a hemacytometer using a pipette. Because polyhedra move about, let the hemacytometer sit for 5–10 min to allow the polyhedra to settle.
2. Examine the hemacytometer under the microscope with a 20× objective on an inverted microscope. The polyhedra in the large gridded central square will be counted (Fig. 2b). Polyhedra appear as small, polyhedral objects in the field of view (Fig. 2c).
3. The central square is divided into 25 smaller squares. Polyhedra in five of these squares that form a diagonal line, as well as any polyhedra that overlap the triple lines that border the top and left sides of the five squares are counted (Fig. 2b). Repeat this for both sides of the hemacytometer and calculate the average number of polyhedra for each individual square by dividing the total number of polyhedra in the 10 small squares by 10.

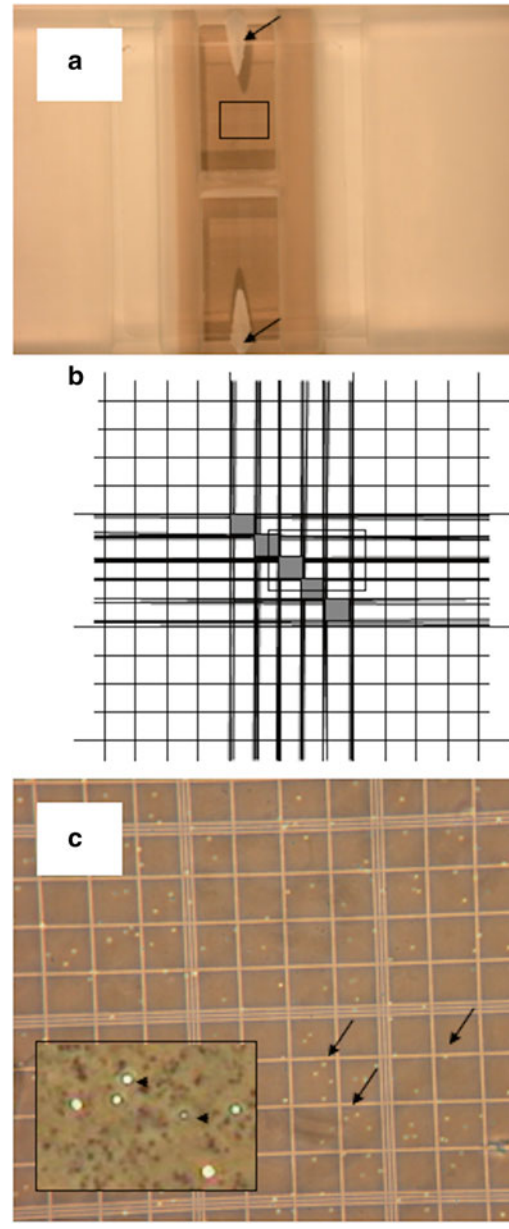


**Fig. 2** Quantification of polyhedra using a hemacytometer. (**a**) Place the coverslip over the center of the hemacytometer as shown and add one drop of virus solution to each groove (*arrows*). The virus solution will spread beneath the coverslip. Detail of the *boxed area* is shown in (**b**). (**b**) Diagram of the grid on each side of the hemacytometer on examination under an inverted microscope. Count the polyhedra in five diagonal squares within the grid (*gray boxes*). Repeat for the other side of the hemacytometer. Detail of the *boxed area* is shown in (**c**). (**c**) Polyhedra (*arrows*) on a hemacytometer grid as seen using an inverted microscope. *Inset*: High magnification image of polyhedra (*arrow heads*)

The concentration in polyhedra/mL can be obtained using the following formula:

$$Y = \text{mean polyhedra per small square} \times 25 \times (\text{total number of squares}) \times \text{dilution factor} \times 10^4,$$

where Y is the concentration in polyhedra/mL.

### 3.3 Droplet Feeding Bioassay for $LC_{50}$ Determination

The droplet feeding bioassay [6, 14] can be used to determine either the median lethal concentration ($LC_{50}$) or median survival time ($ST_{50}$) of baculoviruses in either neonate or early instars [15]. The advantage of this technique is that the virus inoculum is ingested over a short period of time (5–15 min), which is particularly important for determining $ST_{50}$ responses. In this section, the procedures for a $LC_{50}$ bioassay are presented. For estimation of the median lethal dose ($LD_{50}$) using this technique, the amount of fluid ingested must be quantified [16].

#### 3.3.1 Calculation of Range of Concentrations

1. Early instar larvae are generally used for droplet feeding bioassays. After hatch, allow neonate larvae to starve several hours to overnight depending on the species used. If second instar larvae are needed, then place neonate larvae in diet cups with diet. After 1 day at 28 °C, remove the diet and starve the second instar larvae overnight.
2. The concentrations of baculovirus used in droplet feeding bioassays should give a range of mortality from 1 to 99 %. Preliminary bioassays may be required to determine an appropriate range of virus concentrations if the information is not already available. For example, for neonate *H. virescens*, AcMNPV concentrations of $5.0 \times 10^4$, $2.0 \times 10^5$, $5.0 \times 10^5$, $1.0 \times 10^6$, and $2.0 \times 10^6$ polyhedra/mL would be appropriate. For a virus that has not been previously assayed, concentrations increasing by tenfold from $10^3$ to $10^7$ would be appropriate. For dose calculations, these concentrations are designated as M$i$ ($i$ = 1, 2, 3, 4, 5, i.e., M1, M2, M3, M4, and M5).
3. To prepare each viral dose, first determine the concentration (S, polyhedra/μL) of stock polyhedra using a hemacytometer and microscope. Using the formula: Vi (μL) = (175 μL × Mi)/S, to calculate the volume (Vi in μL) of stock polyhedra to make a 175 μL diluted suspension with each desired concentration (Mi). Add a Vi volume of stock polyhedra to a 1.5-mL centrifuge tube containing (128.4-Vi) volume (μL) of $dH_2O$, vortex for seconds to mix it, then add 17.5 μL of blue food coloring (10 % blue color, v/v) and 29.1 μL 33 % (w/v) sucrose solution for palatability. 175 μL of $dH_2O$ with dye and sucrose is used for a negative control treatment.

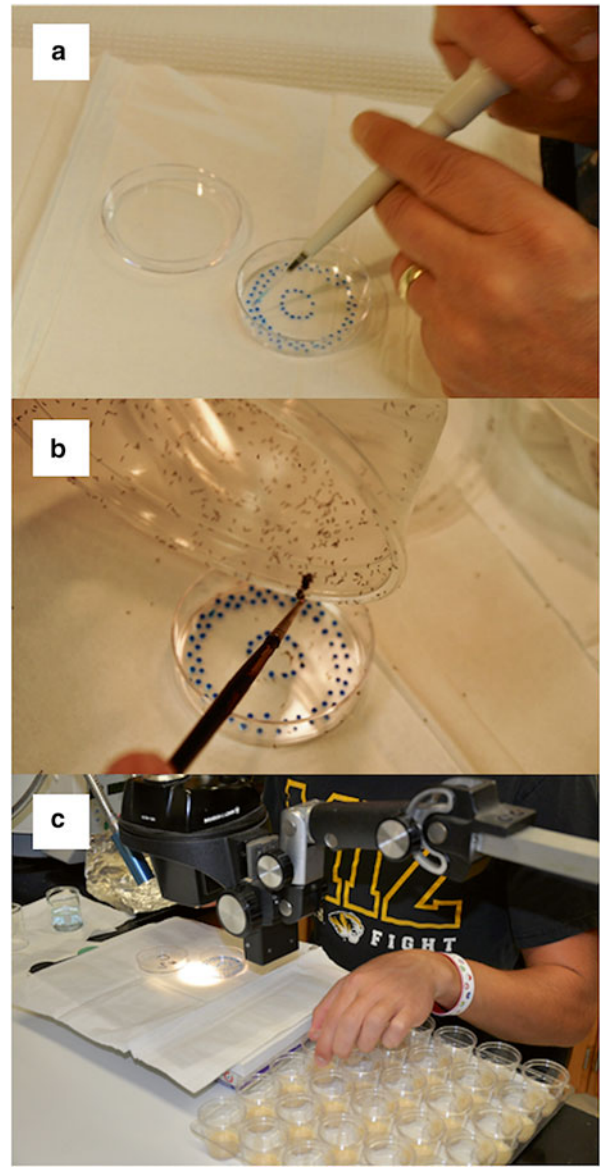


**Fig. 3** Droplet feeding bioassays. (**a**) Concentric circles of droplets of virus solution with blue food dye are used for the droplet feeding bioassay. (**b**) Neonate larvae placed in the center of the dish drink the virus solution and crawl up the sides of the dish. (**c**) Larvae that are identified as having ingested the blue solution under a dissecting scope are transferred to individual diet cups

*3.3.2 Infection by Droplet Feeding*

1. Make concentric circles of small droplets (0.5 μL per droplet) of the virus suspension or negative control in a 60-mm Petri dish using the multidispensing pipette (Fig. 3a).
2. Transfer 40–45 neonates or second instars to the middle of the concentric circles (Fig. 3b). Place the lid on the dish and seal with Parafilm or place a large rubber stopper on the lid to prevent the larvae from escaping.

3. Allow the dish to stand at room temperature for 5–15 min during which time larvae will drink the solution and crawl to the top of the dish. Larvae that have fed can be identified by blue coloration of the anterior gut resulting from the blue food coloring dye. Transfer only the larvae that have fed, each to a 1-oz. plastic cup with a diet prepared as described above. It is helpful to work under a dissecting scope to identify larvae that have ingested virus (Fig. 3c).
4. Cap each cup, place them in a plastic tray, and incubate at 28 °C and 14:10 (L:D) light period.
5. To monitor possible contamination by viruses, it is advised to set up two mock treatments, one at the start and one at the end of the bioassay set-up. On completion of treatments for one virus, change to a new brush and fresh water to transfer larvae.
6. Repeat bioassays three or four times on separate occasions, with at least 30 individuals per dose for each virus or control treatment for lethal concentration bioassays.
7. Check each larva 24 h after infection and eliminate any dead larvae. These early deaths likely result from handling injury. Check the larvae twice daily for the duration of the bioassay, which is usually either 7 or 10 days depending on the speed of the parent virus. Larvae that die from virus infection generally melt when the cup is tapped. However, recombinant viruses do not always cause larvae to melt due to the fast kill rate. Larvae infected with recombinant viruses containing toxins will enter a paralysis before death that is evident when the larvae are examined under a dissecting scope. It is important to recognize the difference between dead and paralyzed larvae in data collection.
8. Record the mortality and time of observation for each tray then calculate $LC_{50}$ values, $LC_{50}$ 95 % confidence limits (CL), and slope with standard error as described in Subheading 3.8.

### 3.4 Diet Plug Bioassay For Determination of Median Lethal Dose

1. The diet plug bioassay with occlusion bodies is usually used for later instars when larvae are large enough to handle (i.e., fourth and fifth instars). Below is the method used for early fourth instars of *H. virescens*.
2. Prepare polyhedra as described in Subheading 3.2. Five or six different concentrations are used with 30 larvae infected at each dose, and repeat bioassay at least three times.
3. The specific virus doses used should be determined by preliminary experiments to obtain a wide range of mortality (1–99 % or narrower) to facilitate probit analysis.
4. Cut *H. virescens* diet into small cubes (~2 $mm^3$) with a scalpel or stainless steel weighing spatula with a tapered end. Transfer diet cubes into individual plastic cups (one cube per cup)

with forceps. If the diet cubes are too small, then they will dry out at low humidity.

5. Add 5 μL polyhedra suspension with food coloring dye (10 %, v/v) to each diet cube. Allow the suspension to soak into the diet for 5–15 min.
6. Transfer one early fourth instar into each cup. Leave larvae in cups overnight and then transfer only those larvae that have completely eaten the diet cube individually to new cups with fresh diet. Starving larvae overnight beforehand will increase the speed with which larvae consume the diet cube (which typically takes around 3 h).
7. Incubate larvae at 28 °C and 14:10 (L:D) light period for 7–10 days depending on the speed of the parent virus being bioassayed. Record the number of dead larvae twice daily.
8. Calculate $LD_{50}$ and 95 % CL, and slope with standard error as described in Subheading 3.8.

### 3.5 Microapplicator-Assisted Bioassays Using Budded Virus

When developed for insecticidal purposes, recombinant baculoviruses usually contain the polyhedrin gene to facilitate oral infection of pest larvae. However, on occasion, there are reasons to test a polyhedrin-negative baculovirus developed primarily for protein expression for potential insecticidal effects. For example, baculoviruses that express proteins that are toxic to cultured insect cells are often of interest. Although the occlusion-derived virus (ODV) of polyhedrin-negative viruses can be tested by oral administration, quantification of ODV is complex. An easier approach is to deliver BV by injection directly into the larval hemocoel using a microapplicator (Fig. 4).

1. The titer of the BV should be determined (titering methodologies are given in other chapters) and appropriate adjustments for a given titer should be made with sterile cell culture medium if necessary. For physiological experiments, injection of $5 \times 10^4$ pfu per fifth instar *H. virescens* would be typical.
2. Load each BV dilution into a plastic tuberculin syringe (1 cc) with a 28.5 (or 32)-gauge needle (sharp tip). The amount of BV solution loaded will depend on the number of larvae to be injected and the amount injected per larva. Usually, 1–3 μL BV solution (with a titer of $10^7$–$10^8$ pfu/mL) can be used for each larva, such that loading 300–500 μL into the syringe is sufficient for most purposes.
3. Set the volume to 1.0 μL for each droplet at the microprocessor control unit. Calibrate the control unit first to ensure that the correct volume is dispensed. The speed that the droplet is dispensed can be set on a scale of 0–9 (where 0 is fast and 9 is slow), with 0–1 commonly used.

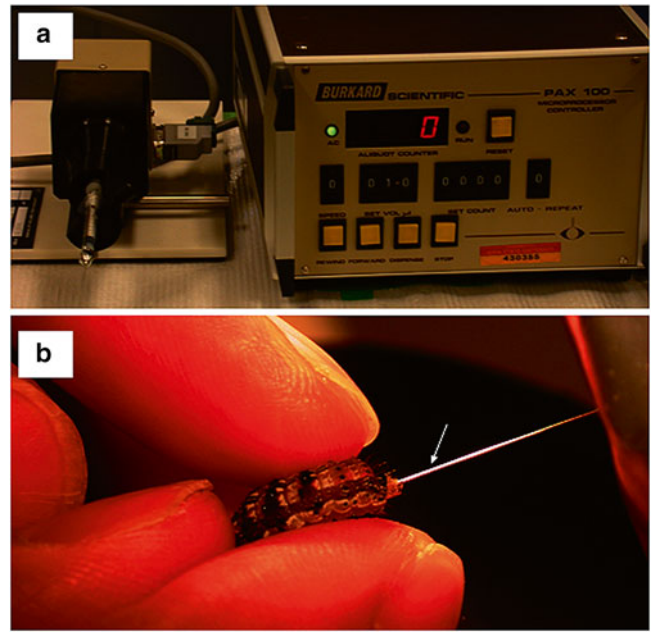


**Fig. 4** Microapplicator assisted bioassays. (**a**) Microapplicator apparatus. The applicator with syringe mount and needle are shown on the *left*, the microprocessor control unit is on the *right*. (**b**) Oral inoculation of fifth instar *H. virescens* using a 32 gauge blunt-tip needle. This same apparatus can be used for injection of larvae with budded virus using a sharp-tip needle (*see* Subheading 3.4)

4. Drive air bubbles out of the syringe by holding the syringe upright to bring air to the needle tip and dispense. Fix the syringe on the syringe support tube. Press the foot switch to test the dispensing system.
5. Early fifth instar larvae are typically injected for *H. virescens*. The larger size of the later instars facilitates the injection process. Insert the needle tip through the planta of one of the larval prolegs and into the body cavity. Press the foot switch to deliver the BV solution into the hemocoel. When the needle tip inserts into the planta of one of the prolegs, change the larval body position or the angle of the insect relative to the needle to avoid damaging the gut and body wall.
6. After injection, return larvae to individual cups with diet and incubate at 28 °C.
7. Late instars do not die from injury to the cuticle resulting from injection. Larvae should be checked 24 h after injection, however, for damage to the gut that could result in death from bacterial infection. This early mortality should be excluded from data analysis (*see* Subheading 3.8.3 for $ST_{50}$ data analysis). Assess larvae twice daily and record deaths.

8. This microapplicator-assisted administration system is also used to deliver polyhedra directly into the midgut for more precise timing and quantification of inoculation for some physiological experiments [10]. When using a microapplicator for oral inoculation of insects with polyhedra, polyhedra are suspended in a neutrally buoyant solution of glycerin and water (3:2, v/v) that is loaded into a plastic or glass tuberculin syringe (1 cc) with a blunt-tip needle (32-gauge) (Fig. 4). Place the syringe on the support tube of the microapplicator. Under a dissecting scope, hold a larva (such as early fourth or fifth instar of *H. virescens*) and insert the needle through the mouth and into the anterior region of the larval midgut where polyhedra are delivered (for a visual demonstration *see* ref. 17).

### 3.6 Droplet Feeding for $ST_{50}$ Determinations

Because different viruses can have different $LC_{50}$s (or $LD_{50}$s), van Beek and Hughes [18] recommended using an $LC_{50}$ and $LC_{99}$ dose to determine the $ST_{50}$. The $LC_{50}$ is essential to determining the $ST_{50}$ because the resulting survival times are relatively insensitive to dose differences. $LC_{50}$ is also important for predicting how a virus will perform in the field because it is unlikely that a baculovirus will be applied at a $LC_{99}$ rate. The $ST_{50}$ can be calculated for all bioassay doses yielding mortalities over 30 %, which gives confidence that the reported $ST_{50}$ is consistent. One assay of larvae infected at an $LC_{99}$ is not sufficient to determine $ST_{50}$.

Survival-time bioassays are performed using droplet feeding for younger instars and a microapplicator for older instars. They can also be performed using a microapplicator to deliver BV derived from a polyhedrin-negative baculovirus directly into the larval hemocoel [19, 20]. Time is an important consideration in these bioassays. After infection, mortality should be recorded every 4–8 h, with more frequent observations when the mortality rate is high. Median survival times ($ST_{50}$) and the 95 % confidence limits are calculated using the Kaplan–Meier Estimator by programs such as SAS and S-PLUS (Tibco Software Inc., Palo Alta, CA, USA). Because the mortality data recorded at different time points for a group of insects are not independent observations, the $ST_{50}$ cannot be calculated using probit analysis that is typically used for calculating $LC_{50}$ or $LD_{50}$. The comparison of multiple $ST_{50}$s is conducted using the log-rank test. A SAS program calculating the $ST_{50}$s is described in Subheading 3.8.3.

### 3.7 Feeding Damage Assay

The amount of feeding damage caused by baculovirus-infected larvae is the most important parameter to consider in terms of crop protection. Feeding assays using leaf material are useful to compare feeding damage caused by recombinant and wild type virus-infected larvae [1]. However, assays performed on individual

leaves may not be wholly representative of what occurs on a plant. For example, if a larva is paralyzed by a neurotoxin and falls from the plant, then the damage sustained by the plant may be less than that caused by a larva housed on a leaf in a Petri dish [21].

1. For feeding assays, *H. virescens* second instar larvae are starved overnight and are mock-infected or infected with a 5× neonate $LC_{99}$ dose of AcMNPV-C6 and AcMLF9.ScathL by the droplet feeding method as described under Subheading 3.3. This dose results in 100 % mortality of second instars.
2. Transfer infected and mock-infected larvae individually to 60-mm-diameter dishes, which contain pieces of organic iceberg lettuce on water-wet filter paper. Place the larvae at 28 °C and 14:10 (L:D) light period.
3. Replace pieces of lettuce every 2–3 days.
4. Measure the areas of the pieces of lettuce with a LI-COR 3100 area meter before and after feeding.
5. After all virus-infected larvae are dead, determine the total area consumed by each larva.
6. Subject the data to one-way analysis of variance (ANOVA) with virus as the main factor.

### 3.8 Data Analysis

Estimates of 50 % are the mainstay for toxicologists who look for potency of a specific chemical ($LC_{50}$ or $LD_{50}$) or non-chemical lethal or sublethal endpoint values. $EC_{50}$ or $ED_{50}$ (effective concentration or dose at 50 %) represent an endpoint where there is no lethal effect (for example the point of 50 % pupation). Most 50 % effects use binomial data (data yielding only two responses such as dead or alive) and the analyses are generally performed the same. The value of 50 % is used because it gives the narrowest confidence limits and can be compared to other 50 % estimations.

This section describes four statistical tests for estimating and comparing 50 % toxicity values for both increasing baculovirus concentration and increasing time: (1) probit analysis (probability unit) to calculate not only $LC_{50}$ but many other LC values, (2) logistic analysis to calculate $LC_{50}$ values and compare $LC_{50}$ values, (3) lifetest analysis to determine the $ST_{50}$, and (4) accelerated life testing to forecast $LC_{50}$ at chronic time points. The first two techniques assume that time is constant. For the third, the SAS program calculates the time it takes to kill 50 % of a population while keeping all other effects constant. The software used for the first three statistical procedures is the SAS statistical package v9.2 or 9.3. The fourth procedure analyzes all concentrations and time points and uses software available from the Society of Toxicology and Chemistry (SETAC).

### 3.8.1 $LC_{50}$ or $LD_{50}$ Determination: The Probit Analysis

The probit analysis was described by Finney [22]. In the following example the mortality from four baculoviruses (labeled A through D) are compared across six concentrations of viruses ranging from $10^4$ to $2.5 \times 10^6$ polyhedra/mL as well as a negative control group ($0 \times 10^0$) from a droplet feeding assay described in Subheading 3.3. Each concentration has 30 individuals that were exposed to baculovirus. The SAS program using the Proc (procedure) Probit program shown below enters the mortality data from the bioassay as virus, concentration (conc), number of dead larvae (mort), and total of larvae tested in each dose (n).

```
data mortality;
input virus conc mort n @@;
datalines;
A 0.00E+00 1 30 A 1.00E+04 2 30 A 2.50E+04 3 29 A 1.00E+05 3 30
A 2.50E+05 23 25 A 1.00E+06 28 30 A 2.50E+06 30 30
B 0.00E+00 1 30 B 1.00E+04 1 30 B 2.50E+04 7 30 B 1.00E+05 11 29
B 2.50E+05 22 30 B 1.00E+06 26 29 B 2.50E+06 29 30
C 0.00E+00 1 30 C 1.00E+04 3 29 C 2.50E+04 13 30 C 1.00E+05 16 29
C 2.50E+05 24 30 C 1.00E+06 30 30 C 2.50E+06 30 30
D 0.00E+00 1 30 D 1.00E+04 3 30 D 2.50E+04 4 30 D 1.00E+05 16 30
D 2.50E+05 15 30 D 1.00E+06 30 30 D 2.50E+06 30 30
;
proc print;
proc sort; by virus:
proc probit log10; by virus;
model mort/n=conc/inversecl;
;
run;
```

The @@ notation at the end of the input statement tells the program to read four values at a time. If this was not present, then virus, conc, mort, and n would need to be on single lines. The Proc Sort and the by virus statements perform a probit analysis for each virus. The output presented below is only a portion of the printout for virus A to conserve space.

```
-------------------------------------------virus=A---------------------------------
This shows the intercept and regression coefficient for virus A along with
other statistics.
                        Standard    95% Confidence      Chi-
     Parameter     DF   Estimate   Error    Limits     Square     Pr       > ChiSq
      Intercept     1   -9.4853    1.1311 -11.7022   -7.2683     70.32     <.0001
      Log10(conc)   1    1.8462    0.2203   1.4144    2.2780     70.23     <.0001
```

Probit Procedure

Probit Analysis on Log10(conc)

| Probability | Log10(conc) | 95% Fiducial | Limits |
|---|---|---|---|
| 0.01 | 3.8776 | 3.4708 | 4.1433 |
| 0.02 | 4.0253 | 3.6593 | 4.2671 |
| 0.03 | 4.1189 | 3.7785 | 4.3462 |
| 0.04 | 4.1894 | 3.8677 | 4.4060 |
| 0.05 | 4.2467 | 3.9401 | 4.4550 |
| 0.06 | 4.2955 | 4.0015 | 4.4968 |
| 0.07 | 4.3383 | 4.0551 | 4.5337 |
| 0.08 | 4.3766 | 4.1030 | 4.5668 |
| 0.09 | 4.4115 | 4.1464 | 4.5971 |
| 0.10 | 4.4435 | 4.1862 | 4.6251 |
| 0.15 | 4.5763 | 4.3496 | 4.7426 |
| 0.20 | 4.6818 | 4.4773 | 4.8382 |
| 0.25 | 4.7723 | 4.5848 | 4.9222 |
| 0.30 | 4.8536 | 4.6794 | 4.9996 |
| 0.35 | 4.9290 | 4.7652 | 5.0732 |
| 0.40 | 5.0004 | 4.8446 | 5.1450 |
| 0.45 | 5.0696 | 4.9196 | 5.2164 |
| **0.50** | **5.1377** | **4.9914** | **5.2886** |
| 0.55 | 5.2057 | 5.0613 | 5.3627 |
| 0.60 | 5.2749 | 5.1305 | 5.4398 |
| 0.65 | 5.3464 | 5.2002 | 5.5214 |
| 0.70 | 5.4217 | 5.2719 | 5.6091 |
| 0.75 | 5.5030 | 5.3474 | 5.7055 |
| 0.80 | 5.5935 | 5.4297 | 5.8147 |
| 0.85 | 5.6991 | 5.5238 | 5.9440 |
| 0.90 | 5.8318 | 5.6398 | 6.1088 |
| 0.91 | 5.8639 | 5.6675 | 6.1490 |
| 0.92 | 5.8987 | 5.6975 | 6.1927 |
| 0.93 | 5.9370 | 5.7304 | 6.2408 |
| 0.94 | 5.9798 | 5.7670 | 6.2948 |
| 0.95 | 6.0286 | 5.8085 | 6.3565 |
| 0.96 | 6.0859 | 5.8571 | 6.4291 |
| 0.97 | 6.1564 | 5.9166 | 6.5187 |
| 0.98 | 6.2501 | 5.9953 | 6.6382 |
| 0.99 | 6.3977 | 6.1187 | 6.8272 |

The above gives a number of LC probability numbers from $LC_1$ to $LC_{99}$ for Virus A. In the middle is the $LC_{50}$ with confidence intervals (bolded). To calculate the $LC_{50}$ and confidence limits take the inverse log of these values to arrive at $1.37 \times 10^5$ ($= 10^{5.1377}$)

polyhedra/mL with confidence intervals of $9.80 \times 10^4$ ($=10^{4.9914}$) and $1.94 \times 10^5$ ($=10^{5.2886}$) polyhedra/mL.

### 3.8.2 Comparing of the LC50 of Two or More Baculoviruses

The following analysis is a modification of a program presented by Stokes et al. [23]. This program computes the $LC_{50}$ for each virus and compares differences between viruses using a parallel line assay. First, the program confirms that there is nonsignificant curvature of the line and then compares the slopes of each virus. If the regression lines prove to be parallel between all compared viruses, then differences between the $LC_{50}$s are compared to test for equality of virus potency using a common slope. Fieller's theorem was first presented to describe the confidence interval for the ratio of two random variables in 1940 [24]. Further readings of Fieller's theorem are given by Read [25], Collet [26], and Zerbe [27]. This section uses the same data as Subheading 3.8.1, but the Proc Logistic procedure is used instead of the Proc Probit procedure in SAS. The SAS program is outlined below with bolded statements that serve as explanations of program statements, but are not necessary to run the program. This program compares the potencies of four baculoviruses in one bioassay, but additional data lines can be added for replications of the bioassay. It can also be modified to compare any number of viruses.

```
Data mortality;
input trt$ conc mort n @@;
datalines;
A 0.00E+00 1 30 A 1.00E+04 2 30 A 2.50E+04 3 29 A 1.00E+05 3 30 A 2.50E+05
  23 25
A 1.00E+06 28 30 A 2.50E+06 30 30
B 0.00E+00 1 30 B 1.00E+04 1 30 B 2.50E+04 7 30 B 1.00E+05 11 29 B 2.50E+05
  22 30
B 1.00E+06 26 29 B 2.50E+06 29 30
C 0.00E+00 1 30 C 1.00E+04 3 29 C 2.50E+04 13 30 C 1.00E+05 16 29 C 2.50E+05
  24 30
C 1.00E+06 30 30 C 2.50E+06 30 30
D 0.00E+00 1 30 D 1.00E+04 3 30 D 2.50E+04 4 30 D 1.00E+05 16 30 D 2.50E+05
  15 30
D 1.00E+06 30 30 D 2.50E+06 30 30
;
proc print;
data assay; set mortality;
*** int_a--int_d are intercepts for the four viruses*******;
*** lconc_a--lconc_d are the slopes for each virus**********;
*** lconc=the overall slope*******************************;
*** sqlconc_a—sqlconc_d, are used to test curvature********;
      int_a=(virus='A');
      int_b=(virus='B');
```

```
      int_c=(virus='C');
      int_d=(virus='D');
      lconc=log10(conc);
      lconc_a=int_a*lconc;
      lconc_b=int_b*lconc;
      lconc_c=int_c*lconc;
      lconc_d=int_d*lconc;
      sqlconc_a=int_a*lconc*lconc;
      sqlconc_b=int_b*lconc*lconc;
      sqlconc_c=int_c*lconc*lconc;
      sqlconc_d=int_d*lconc*lconc;
proc print;
run;
*** Notice that all terms are in the model except lconc.**;
   proc logistic data=assay descending;
      model mort/n = int_a int_b int_c int_d   lconc_a lconc_b lconc_c
                     lconc_d
                     sqlconc_a sqlconc_b  sqlconc_c sqlconc_d   /noint
                     scale=williams aggregate
                     start=14 selection=forward details;
*** The following statements are for testing equality of slopes.**;
      eq_slope: test lconc_a=lconc_b;
      eq_slope: test lconc_a=lconc_c;
      eq_slope: test lconc_a=lconc_d;
      eq_slope: test lconc_b=lconc_c;
      eq_slope: test lconc_b=lconc_d;
      eq_slope: test lconc_c=lconc_d;
   run;
*****The logistic procedure calculates the values for
    LC values, potency values and confidence interval values******;
   proc logistic data=assay descending outest=estimate
                      (drop= intercept _link_ _lnlike_) covout;
      model mort/n = int_a int_b int_c int_d  lconc /
                     noint scale=williams aggregate covb;
   run;
*** PROC IML is a matrix procedure for calculating LC50s and potency values.
    It also
        calculates the fiducial confidence interval for each point
      estimate****************;
   proc iml;
      use estimate;
      start fieller;
```

```
title 'Confidence Intervals';
use estimate;
read all into beta where (_type_='PARMS');
beta=beta`;
read all into cov where (_type_='COV');
ratio=(k`*beta) / (h`*beta);
a=(h`*beta)**2-(3.84)*(h`*cov*h);
b=2*(3.84*(k`*cov*h)-(k`*beta)*(h`*beta));
c=(k`*beta)**2 -(3.84)*(k`*cov*k);
disc=((b**2)-4*a*c);
d=(k`*beta);
e=(k`*cov*k);
if (disc<=0 | a<=0) then do;
print "confidence interval can't be computed", ratio;
stop; end;
sroot=sqrt(disc);
l_b=((-b)-sroot)/(2*a);
u_b=((-b)+sroot)/(2*a);
interval=l_b||u_b;
lname={"l_bound", "u_bound"};
print "95 % ci for ratio based on fieller", ratio interval[colname=lname];
```

***** The following statement tests the equality of slopes between virus A and B.**;**

```
finish fieller;
k={ 1 -1 0 0 0 }`;
h={ 0 0 0 0 1 }`;
run fieller;
```

***** The following statement tests the equality of slopes between virus A and C.**;**

```
k={ 1 0 -1 0 0 }`;
h={ 0 0 0 0 1 }`;
run fieller;
```

***** The following statement tests the equality of slopes between virus A and D.**;**

```
k={ 1 0 0 -1 0 }`;
h={ 0 0 0 0 1 }`;
run fieller;
```

***** The following statement tests the equality of slopes between virus B and C.**;**

```
k={ 0 1 -1 0 0 }`;
h={ 0 0 0 0 1 }`;
run fieller;
```

***** The following statement tests the equality of slopes between virus B and D.**;**

```
     k={ 0 1 0 -1 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
*** The following statement tests the equality of slopes between virus C and
    D.**;
     k={ 0 0 1 -1 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
*** The following statement calculates the LC50 of Virus A.**;
     k={-1 0 0 0 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
*** The following statement calculates the LC50 of Virus B.**;
     k={0 -1 0 0 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
*** The following statement calculates the LC50 of Virus C.**;
     k={0 0 -1 0 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
*** The following statement calculates the LC50 of Virus D.**;
     k={0 0 0 -1 0 }`;
     h={ 0 0 0 0 1 }`;
     run fieller;
```

The highlighted section below displays the results of the tests for the significance for the quadratic terms. The nonsignificant result (>0.05) confirms that there is no curvature in the line and that the test can proceed with the regression analysis.

The LOGISTIC Procedure

Analysis of Maximum Likelihood Estimates

| Parameter | DF | Standard Estimate | Error | Wald Chi-Square | Pr > ChiSq |
|---|---|---|---|---|---|
| int_a | 1 | 23.4501 | 39.0065 | 0.3614 | 0.5477 |
| int_b | 1 | -13.0908 | 20.5851 | 0.4044 | 0.5248 |
| int_c | 1 | 4.7502 | 26.0195 | 0.0333 | 0.8551 |
| int_d | 1 | 13.9770 | 25.2510 | 0.3064 | 0.5799 |
| lconc_a | 1 | -13.4109 | 16.2411 | 0.6818 | 0.4090 |
| lconc_b | 1 | 2.7005 | 8.0693 | 0.1120 | 0.7379 |
| lconc_c | 1 | -4.3975 | 10.8874 | 0.1631 | 0.6863 |
| lconc_d | 1 | -8.4543 | 10.3975 | 0.6611 | 0.4162 |
| sqlconc_a | 1 | 1.7086 | 1.6790 | 1.0356 | 0.3089 |
| sqlconc_b | 1 | -0.0232 | 0.7841 | 0.0009 | 0.9764 |
| sqlconc_c | 1 | 0.7075 | 1.1287 | 0.3930 | 0.5307 |
| sqlconc_d | 1 | 1.1110 | 1.0615 | 1.0954 | 0.2953 |

Below are the tests for equality of slopes in the order of Virus A*B, A*C, A*D, B*C, B*D, and C*D. In this example, all of the slopes are equal.

Linear Hypotheses Testing Results

| Label | Wald Chi-Square | DF | Pr > ChiSq |
|---|---|---|---|
| eq_slope | 0.7893 | 1 | 0.3743 |
| eq_slope | 0.2125 | 1 | 0.6448 |
| eq_slope | 0.0661 | 1 | 0.7972 |
| eq_slope | 0.2743 | 1 | 0.6004 |
| eq_slope | 0.7183 | 1 | 0.3967 |
| eq_slope | 0.0726 | 1 | 0.7876 |

Since the slopes are equal, a common slope is used (lconc) for further comparisons.

The LOGISTIC Procedure

Analysis of Maximum Likelihood Estimates

| Parameter | DF | Estimate | Standard Error | Wald Chi-Square | Pr > ChiSq |
|---|---|---|---|---|---|
| int_a | 1 | -14.0290 | 1.5249 | 84.6402 | <.0001 |
| int_b | 1 | -13.8340 | 1.5084 | 84.1104 | <.0001 |
| int_c | 1 | -12.9157 | 1.4292 | 81.6684 | <.0001 |
| int_d | 1 | -13.8022 | 1.5057 | 84.0299 | <.0001 |
| lconc | 1 | 2.7264 | 0.2889 | 89.0312 | <.0001 |

Estimated Covariance Matrix

| Parameter | int_a | int_b | int_c | int_d | lconc |
|---|---|---|---|---|---|
| int_a | 2.32529 | 2.190323 | 2.067215 | 2.186433 | -0.42999 |
| int_b | 2.190323 | 2.275339 | 2.044638 | 2.162554 | -0.42529 |
| int_c | 2.067215 | 2.044638 | 2.042603 | 2.041008 | -0.40139 |
| int_d | 2.186433 | 2.162554 | 2.041008 | 2.267053 | -0.42453 |
| lconc | -0.42999 | -0.42529 | -0.40139 | -0.42453 | 0.083489 |

Below are the results of the Proc Iml (interactive matrix language) procedure. The first six ratios and confidence intervals represent the potencies of one virus versus another beginning with virus A versus virus B and ending with virus C versus virus D. For example, the potency of virus A versus virus B is calculated as (int_a/lconc)-(int_b/lconc). The last four ratios and confidence intervals represent the $LC_{50}$s for virus A through virus D. For example, the $LC_{50}$ of virus A is int_a/lconc. This $LC_{50}$ should closely match with the previous $LC_{50}$ found using the probit analysis in Subheading 3.8.1. If the potencies between two viruses are equal, then the confidence intervals will include 0. To calculate the $LC_{50}$ and ratios from the generated numbers, the antilog of each

value must be taken. If the lower and upper bounds (confidence intervals) of each potency comparison are equal, then the confidence intervals will include 1 (i.e., the antilog of 0). The program output data are shown in Table 1 after the antilogs of the ratios and lower and upper bounds are calculated to determine the $LC_{50}$s and confidence limits.

```
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  -0.071523 -0.416647 0.2722237
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  -0.408335 -0.759092 -0.063508
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  -0.083197 -0.427975 0.2599678
```

**Table 1**
**Calculation of $LC_{50}$ from SAS printout**

| Potency | Ratio | Confidence intervals | | Antilog of ratio | Confidence intervals | | Significant difference? |
|---|---|---|---|---|---|---|---|
| | | Lower | Upper | | Lower | Upper | |
| *Comparison of ratios* | | | | | | | |
| A*B | −0.071523 | −0.416647 | 0.2722237 | 0.8482 | 0.3831 | 1.8716 | No |
| A*C | −0.408335 | −0.759092 | −0.063508 | 0.3905 | 0.1741 | 0.8640 | Yes |
| A*D | −0.083197 | −0.427975 | 0.2599678 | 0.8257 | 0.3733 | 1.8196 | No |
| B*C | −0.336812 | −0.685325 | 0.0071483 | 0.4605 | 0.2064 | 1.0166 | No |
| B*D | −0.011673 | −0.354291 | 0.3307074 | 0.9735 | 0.4423 | 2.1414 | No |
| C*D | 0.3251384 | −0.018455 | 0.6730479 | 2.1142 | 0.9584 | 4.7103 | No |
| *LC50s* | | | | | | | |
| A | 5.1456499 | 4.9008819 | 5.3900088 | $1.40\times10^5$ | $7.96\times10^4$ | $2.45\times10^5$ | |
| B | 5.0741267 | 4.8306784 | 5.3157886 | $1.19\times10^5$ | $6.77\times10^4$ | $2.07\times10^5$ | |
| C | 4.7373149 | 4.4868032 | 4.9814872 | $5.46\times10^4$ | $3.07\times10^4$ | $9.58\times10^4$ | |
| D | 5.0624533 | 4.8195436 | 5.3033397 | $1.15\times10^5$ | $6.60\times10^4$ | $2.01\times10^5$ | |

```
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  -0.336812 -0.685325 0.0071483
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  -0.011673 -0.354291 0.3307074
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  0.3251384 -0.018455 0.6730479
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  5.1456499 4.9008819 5.3900088
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  5.0741267 4.8306784 5.3157886
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  4.7373149 4.4868032 4.9814872
95 % ci for ratio based on fieller
                interval
      ratio    l_bound    u_bound
  5.0624533 4.8195436 5.303339
```

### 3.8.3 $ST_{50}$ Calculation Using Life Testing

Life testing calculates the amount of time it takes to reach an event. An event can have different end points such as death or the time a particular instar is reached. If all the subjects die, then a simple regression can be performed. Many times an experiment is terminated before all of the experimental units have died. Does the experimenter just delete these data? The answer is no. If the subjects do not reach an endpoint at the time the experiment has ended, then it is referred to as censored data. SAS uses a procedure referred to as life testing that incorporates censored data. These data cannot be deleted because a bias will exist when comparing one treatment versus another if one treatment has more censored data than another. The solution was presented by Kaplan and Meier [28] and several books describe the survival analysis methodology [29, 30].

One of the main procedures in the SAS statistical package is the Proc Lifetest procedure. Below is the Proc Lifetest program that was used to calculate the $ST_{50}$s and to compare differences between two virus $ST_{50}$s. The data were generated from two groups of insects infected with virus A or B with an overall mortality of 82.3 and 83.3 %, respectively.

```
.data time;
input Virus$ Tm status;
datalines;
A       44.8       0
A       44.8       0
A       44.8       0
A       44.8       0
A       44.8       0
A       44.8       0
A       44.8       0
A       49.3       0
A       49.3       0
A       57.9       0
A       57.9       0
A       57.9       0
A       57.9       0
A       57.9       0
A       57.9       0
A       67.4       0
A       67.4       0
A       67.4       0
A       67.4       0
A       78.3       0
A       164.1       0
A       164.1       0
A       164.1       0
A       164.1       0
A       164.1       1
A       164.1       1
A       164.1       1
A       164.1       1
A       164.1       1
B       61.3       0
B       61.3       0
B       61.3       0
B       61.3       0
```

```
B      76.7      0
B      76.7      0
B      76.7      0
B      76.7      0
B      76.7      0
B      81.8      0
B      81.8      0
B      81.8      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      96.4      0
B      119.4      0
B      167.3      0
B      167.3      0
B      167.3      0
B      167.3      1
B      167.3      1
B      167.3      1
B      167.3      1
B      167.3      1
proc print;
title 'Survival Probability versus Survival Time';
symbol1 l=1 c=black ; symbol2 l=2 c=black;
proc lifetest plots=(s,ls);
time tm*status(1);
strata virus;
run;
```

This program inputs three data items per line for censoring: (1) virus identification, (2) time of death and (3) data input. The censoring code 0 indicates that the larvae died, while a code of 1 means that the larva was still alive at the end of the experiment. Note that there were several 0s at the last inspection time. This means that the experimental unit died on the last day of the experiment and therefore it is not censored. Below is an abbreviated form of the output.

Survival Probability versus Survival Time

Quartile Estimates for Virus A

| Percent | Point Estimate | Transform | 95% Confidence Interval [Lower | Upper) |
|---|---|---|---|---|
| 75 | 164.100 | LOGLOG | 67.400 | . |
| 50 | 57.900 | LOGLOG | 49.300 | 78.300 |
| 25 | 49.300 | LOGLOG | 44.800 | 57.900 |

| Mean | Standard Error |
|---|---|
| 89.117 | 9.670 |

Quartile Estimates for Virus B

| Percent | Point Estimate | Transform | 95% Confidence Interval [Lower | Upper) |
|---|---|---|---|---|
| 75 | 167.300 | LOGLOG | 96.400 | . |
| 50 | 96.400 | LOGLOG | 81.800 | 96.400 |
| 25 | 76.700 | LOGLOG | 61.300 | 81.800 |

| Mean | Standard Error |
|---|---|
| 106.650 | 7.225 |

The LIFETEST Procedure

Summary of the Number of Censored and Uncensored Values

| Stratum | Virus | Total | Failed | Censored | Percent Censored |
|---|---|---|---|---|---|
| 1 | A | 29 | 24 | 5 | 17.24 |
| 2 | B | 30 | 25 | 5 | 16.67 |
| Total | | 59 | 49 | 10 | 16.95 |

Test of Equality over Strata

| Test | Chi-Square | DF | Pr > Chi-Square |
|---|---|---|---|
| Log-Rank | 4.5046 | 1 | 0.0338 |
| Wilcoxon | 9.1546 | 1 | 0.0025 |
| -2Log(LR) | 0.3642 | 1 | 0.5462 |

This analysis indicates that virus A had an $ST_{50} = 57.9$ h and virus B had an $ST_{50} = 96.4$ h. If the data distributions are not known, then the best indicator of significance is the Log-Rank test (Kaplan–Meier test) where a chi-square value <0.05 indicates that the two $ST_{50}$s are significantly different. In the above example the Log-Rank chi-square value is 0.0338, thereby indicating a significant difference.

### *3.8.4 Estimating $LC_{50}$ Using Time of Inspection and Concentration Simultaneously*

Many acute toxicity tests using binomial data not only have different concentration levels but also different evaluation or inspection times. However, many times only the $LC_{50}$ values are presented at one inspection time. Software is available from SETAC (Society of

Toxicology and Chemistry) that can use all concentrations and inspection time points [8] and is based on the theory of accelerated life testing [31, 32]. In other words, it accelerates the time at which an experimental unit dies due to the concentration of some factor, e.g., virus. The method was originally used for mechanical and electrical devices placed under a short-term stress (e.g., a generator running constantly at full power and high heat) to predict long-term time to fail. In this software the model is applied to organisms under stress (i.e., virus) and the variable measured is time to death. The model assumes that both exposure concentration and time duration affect survival probability, and can be summarized over the entire concentration-time-response data of a toxicity test. Actual proportion responses are used, but probit transformations are not.

Accelerated life testing allows the use of all bioassay data simultaneously in a multiple regression (surface response) approach. Because both concentration (or dose) and time values are present in the same survival function, the percent effect ($LC_{50}$) can be forecasted beyond the last observation time. Below is the data input for three replications of a bioassay of one virus with inspections times, viral concentration, number of larvae tested, and number of dead larvae. The time has been averaged to the middle of each inspection time.

| log10 concentration | Inspection time (h) | Total tested | Accumulative dead |
|---|---|---|---|
| 0 | 24 | 30 | 0 |
| 4 | 24 | 30 | 0 |
| 4.4 | 24 | 30 | 0 |
| 5 | 24 | 29 | 0 |
| 5.4 | 24 | 30 | 0 |
| 6 | 24 | 29 | 0 |
| 6.4 | 24 | 30 | 0 |
| 0 | 51 | 30 | 0 |
| 4 | 51 | 30 | 0 |
| 4.4 | 51 | 30 | 0 |
| 5 | 51 | 29 | 0 |
| 5.4 | 51 | 30 | 0 |
| 6 | 51 | 29 | 0 |
| 6.4 | 51 | 30 | 0 |
| 0 | 70 | 30 | 0 |
| 4 | 70 | 30 | 0 |

| | | | |
|---|---|---|---|
| 4.4 | 70 | 30 | 0 |
| 5 | 70 | 29 | 0 |
| 5.4 | 70 | 30 | 0 |
| 6 | 70 | 29 | 1 |
| 6.4 | 70 | 30 | 1 |
| 0 | 78 | 30 | 0 |
| 4 | 78 | 30 | 0 |
| 4.4 | 78 | 30 | 0 |
| 5 | 78 | 29 | 1 |
| 5.4 | 78 | 30 | 0 |
| 6 | 78 | 29 | 1 |
| 6.4 | 78 | 30 | 1 |
| 0 | 83 | 30 | 0 |
| 4 | 83 | 30 | 0 |
| 4.4 | 83 | 30 | 0 |
| 5 | 83 | 29 | 1 |
| 5.4 | 83 | 30 | 0 |
| 6 | 83 | 29 | 1 |
| 6.4 | 83 | 30 | 1 |
| 0 | 95 | 30 | 0 |
| 4 | 95 | 30 | 0 |
| 4.4 | 95 | 30 | 1 |
| 5 | 95 | 29 | 3 |
| 5.4 | 95 | 30 | 4 |
| 6 | 95 | 29 | 12 |
| 6.4 | 95 | 30 | 15 |
| 0 | 101 | 30 | 0 |
| 4 | 101 | 30 | 0 |
| 4.4 | 101 | 30 | 2 |
| 5 | 101 | 29 | 3 |
| 5.4 | 101 | 30 | 6 |
| 6 | 101 | 29 | 15 |
| 6.4 | 101 | 30 | 19 |
| 0 | 106 | 30 | 0 |

| | | | |
|---|---|---|---|
| 4 | 106 | 30 | 0 |
| 4.4 | 106 | 30 | 6 |
| 5 | 106 | 29 | 6 |
| 5.4 | 106 | 30 | 15 |
| 6 | 106 | 29 | 22 |
| 6.4 | 106 | 30 | 26 |
| 0 | 119 | 30 | 0 |
| 4 | 119 | 30 | 0 |
| 4.4 | 119 | 30 | 6 |
| 5 | 119 | 29 | 11 |
| 5.4 | 119 | 30 | 22 |
| 6 | 119 | 29 | 26 |
| 6.4 | 119 | 30 | 27 |
| 0 | 126 | 30 | 0 |
| 4 | 126 | 30 | 1 |
| 4.4 | 126 | 30 | 6 |
| 5 | 126 | 29 | 11 |
| 5.4 | 126 | 30 | 22 |
| 6 | 126 | 29 | 26 |
| 6.4 | 126 | 30 | 28 |
| 0 | 143 | 30 | 0 |
| 4 | 143 | 30 | 1 |
| 4.4 | 143 | 30 | 7 |
| 5 | 143 | 29 | 11 |
| 5.4 | 143 | 30 | 22 |
| 6 | 143 | 29 | 26 |
| 6.4 | 143 | 30 | 29 |
| 0 | 149 | 30 | 0 |
| 4 | 149 | 30 | 1 |
| 4.4 | 149 | 30 | 7 |
| 5 | 149 | 29 | 11 |
| 5.4 | 149 | 30 | 22 |
| 6 | 149 | 29 | 26 |
| 6.4 | 149 | 30 | 29 |

| | | | |
|---|---|---|---|
| 0 | 167 | 30 | 0 |
| 4 | 167 | 30 | 1 |
| 4.4 | 167 | 30 | 7 |
| 5 | 167 | 29 | 11 |
| 5.4 | 167 | 30 | 22 |
| 6 | 167 | 29 | 26 |
| 6.4 | 167 | 30 | 29 |

Notice that the data were inspected at 13 different times. The following is the output from the accelerated life testing procedure.

24-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 8.88986 | 0.22947 | 8.44009 | 9.33963 |
| 20.00% | 9.67733 | 0.11925 | 9.44360 | 9.91107 |
| 30.00% | 10.20454 | 0.28226 | 9.65131 | 10.75777 |
| 40.00% | 10.62766 | 0.38138 | 9.88015 | 11.37516 |
| 50.00% | 11.00093 | 0.46257 | 10.09431 | 11.90756 |
| 60.00% | 11.35373 | 0.53691 | 10.30140 | 12.40605 |
| 70.00% | 11.70983 | 0.61084 | 10.51259 | 12.90706 |
| 80.00% | 12.10063 | 0.69152 | 10.74527 | 13.45599 |
| 90.00% | 12.60086 | 0.79487 | 11.04293 | 14.15879 |

51-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 6.54140 | 0.21353 | 6.12288 | 6.95993 |
| 20.00% | 7.12085 | 0.19764 | 6.73348 | 7.50822 |
| 30.00% | 7.50878 | 0.16518 | 7.18503 | 7.83253 |
| 40.00% | 7.82012 | 0.11373 | 7.59720 | 8.04305 |
| 50.00% | 8.09479 | 0.06059 | 7.97603 | 8.21355 |
| 60.00% | 8.35438 | 0.15170 | 8.05704 | 8.65173 |
| 70.00% | 8.61641 | 0.21530 | 8.19441 | 9.03841 |
| 80.00% | 8.90398 | 0.27685 | 8.36136 | 9.44660 |
| 90.00% | 9.27206 | 0.35069 | 8.58470 | 9.95941 |

70-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 10.00% | 5.75045 | 0.15579 | 5.44510 | 6.05580 |
| 20.00% | 6.25983 | 0.16062 | 5.94501 | 6.57465 |
| 30.00% | 6.60086 | 0.14896 | 6.30890 | 6.89282 |
| 40.00% | 6.87455 | 0.12714 | 6.62536 | 7.12374 |
| 50.00% | 7.11601 | 0.09108 | 6.93749 | 7.29452 |
| 60.00% | 7.34421 | 0.03899 | 7.26778 | 7.42064 |
| 70.00% | 7.57456 | 0.11805 | 7.34317 | 7.80594 |
| 80.00% | 7.82735 | 0.17507 | 7.48420 | 8.17050 |
| 90.00% | 8.15093 | 0.23893 | 7.68263 | 8.61922 |

78-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 5.50270 | 0.12939 | 5.24909 | 5.75630 |
| 20.00% | 5.99013 | 0.14078 | 5.71419 | 6.26607 |
| 30.00% | 6.31647 | 0.13402 | 6.05379 | 6.57914 |
| 40.00% | 6.57837 | 0.11747 | 6.34812 | 6.80862 |
| 50.00% | 6.80942 | 0.08910 | 6.63479 | 6.98406 |
| 60.00% | 7.02780 | 0.01875 | 6.99104 | 7.06455 |
| 70.00% | 7.24822 | 0.09732 | 7.05747 | 7.43897 |
| 80.00% | 7.49012 | 0.15141 | 7.19336 | 7.78688 |
| 90.00% | 7.79975 | 0.21099 | 7.38621 | 8.21329 |

83-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 5.36530 | 0.11169 | 5.14639 | 5.58422 |
| 20.00% | 5.84057 | 0.12742 | 5.59083 | 6.09031 |
| 30.00% | 6.15875 | 0.12328 | 5.91713 | 6.40038 |
| 40.00% | 6.41412 | 0.10923 | 6.20002 | 6.62821 |
| 50.00% | 6.63940 | 0.08394 | 6.47486 | 6.80394 |
| 60.00% | 6.85232 | 0.02354 | 6.80618 | 6.89847 |
| 70.00% | 7.06724 | 0.08937 | 6.89208 | 7.24241 |
| 80.00% | 7.30310 | 0.14078 | 7.02718 | 7.57903 |
| 90.00% | 7.60501 | 0.19746 | 7.21799 | 7.99202 |

95-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 10.00% | 5.07841 | 0.05885 | 4.96306 | 5.19376 |
| 20.00% | 5.52826 | 0.09093 | 5.35003 | 5.70649 |
| 30.00% | 5.82943 | 0.09287 | 5.64739 | 6.01147 |
| 40.00% | 6.07114 | 0.08325 | 5.90797 | 6.23431 |
| 50.00% | 6.28438 | 0.06157 | 6.16370 | 6.40505 |
| 60.00% | 6.48591 | 0.02693 | 6.43313 | 6.53870 |
| 70.00% | 6.68934 | 0.08324 | 6.52617 | 6.85251 |
| 80.00% | 6.91259 | 0.12563 | 6.66635 | 7.15883 |
| 90.00% | 7.19835 | 0.17457 | 6.85618 | 7.54051 |

101-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 4.95340 | 0.02161 | 4.91103 | 4.99576 |
| 20.00% | 5.39218 | 0.06814 | 5.25861 | 5.52574 |
| 30.00% | 5.68593 | 0.07403 | 5.54082 | 5.83104 |
| 40.00% | 5.92169 | 0.06596 | 5.79241 | 6.05097 |
| 50.00% | 6.12968 | 0.04304 | 6.04532 | 6.21404 |
| 60.00% | 6.32626 | 0.04388 | 6.24024 | 6.41227 |
| 70.00% | 6.52467 | 0.08514 | 6.35779 | 6.69156 |
| 80.00% | 6.74243 | 0.12235 | 6.50262 | 6.98224 |
| 90.00% | 7.02115 | 0.16717 | 6.69351 | 7.34880 |

106-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 4.85694 | 0.05905 | 4.74120 | 4.97269 |
| 20.00% | 5.28718 | 0.04245 | 5.20398 | 5.37038 |
| 30.00% | 5.57522 | 0.05440 | 5.46858 | 5.68186 |
| 40.00% | 5.80638 | 0.04710 | 5.71406 | 5.89870 |
| 50.00% | 6.01032 | 0.01298 | 5.98487 | 6.03578 |
| 60.00% | 6.20307 | 0.05558 | 6.09413 | 6.31201 |
| 70.00% | 6.39763 | 0.08824 | 6.22466 | 6.57059 |
| 80.00% | 6.61114 | 0.12124 | 6.37351 | 6.84877 |
| 90.00% | 6.88444 | 0.16260 | 6.56573 | 7.20314 |

119-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 10.00% | 4.63358 | 0.10195 | 4.43376 | 4.83340 |
| 20.00% | 5.04403 | 0.06981 | 4.90720 | 5.18086 |
| 30.00% | 5.31882 | 0.05594 | 5.20917 | 5.42847 |
| 40.00% | 5.53936 | 0.05584 | 5.42990 | 5.64881 |
| 50.00% | 5.73392 | 0.06541 | 5.60571 | 5.86212 |
| 60.00% | 5.91780 | 0.08054 | 5.75994 | 6.07567 |
| 70.00% | 6.10341 | 0.09954 | 5.90830 | 6.29852 |
| 80.00% | 6.30710 | 0.12311 | 6.06580 | 6.54840 |
| 90.00% | 6.56783 | 0.15602 | 6.26204 | 6.87362 |

126-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 4.52704 | 0.11672 | 4.29826 | 4.75582 |
| 20.00% | 4.92805 | 0.08987 | 4.75190 | 5.10421 |
| 30.00% | 5.19652 | 0.07827 | 5.04311 | 5.34994 |
| 40.00% | 5.41199 | 0.07633 | 5.26238 | 5.56160 |
| 50.00% | 5.60208 | 0.08143 | 5.44247 | 5.76168 |
| 60.00% | 5.78173 | 0.09172 | 5.60196 | 5.96150 |
| 70.00% | 5.96307 | 0.10633 | 5.75465 | 6.17149 |
| 80.00% | 6.16208 | 0.12593 | 5.91525 | 6.40891 |
| 90.00% | 6.41682 | 0.15480 | 6.11340 | 6.72023 |

143-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 4.29977 | 0.14255 | 4.02037 | 4.57917 |
| 20.00% | 4.68065 | 0.12198 | 4.44156 | 4.91974 |
| 30.00% | 4.93564 | 0.11239 | 4.71535 | 5.15594 |
| 40.00% | 5.14029 | 0.10887 | 4.92690 | 5.35368 |
| 50.00% | 5.32084 | 0.10972 | 5.10578 | 5.53590 |
| 60.00% | 5.49147 | 0.11426 | 5.26751 | 5.71543 |
| 70.00% | 5.66371 | 0.12244 | 5.42372 | 5.90369 |
| 80.00% | 5.85273 | 0.13513 | 5.58788 | 6.11758 |
| 90.00% | 6.09468 | 0.15604 | 5.78883 | 6.40052 |

149-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 10.00% | 4.22845 | 0.14952 | 3.93539 | 4.52151 |
| 20.00% | 4.60301 | 0.13034 | 4.34753 | 4.85848 |
| 30.00% | 4.85377 | 0.12119 | 4.61623 | 5.09132 |
| 40.00% | 5.05503 | 0.11745 | 4.82483 | 5.28523 |
| 50.00% | 5.23258 | 0.11757 | 5.00213 | 5.46302 |
| 60.00% | 5.40038 | 0.12099 | 5.16323 | 5.63754 |
| 70.00% | 5.56976 | 0.12774 | 5.31938 | 5.82014 |
| 80.00% | 5.75564 | 0.13870 | 5.48380 | 6.02749 |
| 90.00% | 5.99358 | 0.15738 | 5.68510 | 6.30205 |

167-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 4.03667 | 0.16624 | 3.71084 | 4.36251 |
| 20.00% | 4.39425 | 0.15020 | 4.09985 | 4.68865 |
| 30.00% | 4.63364 | 0.14210 | 4.35511 | 4.91217 |
| 40.00% | 4.82577 | 0.13811 | 4.55507 | 5.09646 |
| 50.00% | 4.99526 | 0.13697 | 4.72679 | 5.26374 |
| 60.00% | 5.15546 | 0.13830 | 4.88439 | 5.42652 |
| 70.00% | 5.31716 | 0.14216 | 5.03852 | 5.59580 |
| 80.00% | 5.49461 | 0.14936 | 5.20186 | 5.78736 |
| 90.00% | 5.72175 | 0.16284 | 5.40258 | 6.04092 |

240-Hours

| Mortality | Concentration | Standard Error | 95.00% lower limit | 95.00% upper limit |
|---|---|---|---|---|
| 10.00% | 3.48280 | 0.20212 | 3.08665 | 3.87895 |
| 20.00% | 3.79131 | 0.19289 | 3.41324 | 4.16939 |
| 30.00% | 3.99786 | 0.18766 | 3.63003 | 4.36568 |
| 40.00% | 4.16362 | 0.18440 | 3.80219 | 4.52506 |
| 50.00% | 4.30986 | 0.18245 | 3.95226 | 4.66747 |
| 60.00% | 4.44808 | 0.18157 | 4.09220 | 4.80395 |
| 70.00% | 4.58759 | 0.18177 | 4.23131 | 4.94386 |
| 80.00% | 4.74069 | 0.18340 | 4.38122 | 5.10017 |
| 90.00% | 4.93667 | 0.18783 | 4.56851 | 5.30482 |

Note that the last inspection time in the data set was at 167 h. This program can project the $LC_{50}$ beyond the data collection period. For example, at 240 h the $LC_{50}$ is predicted to be 4.30986

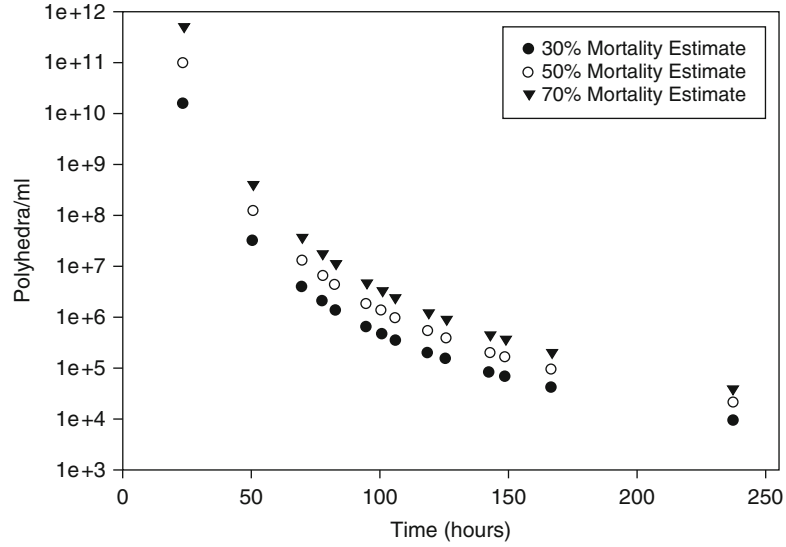


**Fig. 5** Plot of Estimated $LC_{30}$, $LC_{50}$, and $LC_{70}$ values at various check times calculated by accelerated life testing

on the $\log_{10}$ scale or $2.04 \times 10^4$ ($=10^{4.30986}$) polyhedra/mL. The predicted $LC_{30}$, $LC_{50}$, and $LC_{70}$ over time from this example are graphed in Fig. 5. The utility of this program is that it calculates the virus concentration or dose needed to reach an $LC_{50}$ at any given time. This information can be generated for each larval instar and, with a little practice, the control rate predicted for field application.

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# Part VII

## Miscellaneous Techniques and Applications of the Baculovirus/Insect Cell System

# Chapter 22

# Evaluating Baculovirus Infection Using Green Fluorescent Protein and Variants

**Hsuan-Chen Wu, Hyung Joon Cha, and William E. Bentley**

## Abstract

By use of a strategy incorporating the green fluorescent protein (GFP), facile and rapid monitoring and visualization of baculovirus infection in insect cells is possible in vivo. This chapter describes two techniques for simple determination of virus titer in the baculovirus expression system using GFP co-expression and rapid monitoring of Sf-9 insect cell infection using a combination of GFP and the early-to-late (ETL) promoter of the virus vector. Because of its early appearance, GFP, when placed under the control of ETL promoter, will facilitate vector construction, virus isolation, and titer determination.

**Key words** Baculovirus infection, Monitoring, Green fluorescent protein, Insect cell culture

## 1 Introduction

It is important to know the titer of a recombinant baculovirus stock, expressed in plaque-forming units (pfu/mL), when preparing new virus stocks or when carrying out infections for protein production. The productivity of the baculovirus expression vector system (BEVS) is sensitive to cell density, viability, nutrient levels, and the multiplicity of infection (MOI), which is the ratio of added infectious virus particles to viable cells at the time of infection. Thus, it is important to know both the viable cell concentration in the bioreactor and the infectious virus particle concentration in the virus stock. With the virus titer and the viable cell number, one can optimize production of the desired protein. The viable cell number can be determined by hemacytometer counting using Trypan Blue staining [1]. The titer of a recombinant baculovirus stock is commonly determined either by plaque assay [2] (also *see* Chapter 4) or by end-point dilution [1] (also *see* Chapter 10). Other methods of titering a baculovirus stock are given in Chapters 5 and 11. While the plaque assay has the potential to accurately determine the virus titer, it is difficult to perform and requires a long processing time (up to ~1 week). Furthermore, visualizing plaques can be difficult.

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While the end-point dilution method is easier to set up, the results are often more difficult to interpret than plaque assays, particularly when titrating recombinant virus stocks. Wild-type virus is easily detected because of the accumulation of its occlusion bodies, but recombinant virus infection can sometimes be difficult to detect because of the lack of occlusion bodies. To distinguish between infected and uninfected cells, a decrease in cell density and an increase in cell size [3] are monitored (with difficulty) by regular light microscopy. To enhance the existing titration methods, alternative techniques for baculovirus titration have continued to emerge [4–9]. One strategy incorporates heterologous reporter proteins for the detection and purification of proteins upon infection by baculovirus [10–15]. For example, Sussman [12] introduced the bacterial *E. coli* β-galactosidase (β-gal) gene (*lacZ*) into transfer vectors for enhancing assay efficacy and simplicity. Thereafter, many commercially available transfer vectors contained this marker gene. However, the use of this marker requires an additional expensive substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal]), which must be added in an additional step near the end of the assay. On the other hand, fluorescent reporter proteins, which can be easily observed upon chromophore excitation [16, 17], are equally promising and simpler. By incorporating the gene for a fluorescent protein (e.g., green fluorescent protein, GFP) instead of *lacZ* into the baculovirus, a marker can be created for visualizing gene expression with no need for a substrate or other cofactors.

GFP, one of the most popular fluorescence-based reporters used in biological and medical research [18], was originally isolated from the jellyfish, *Aequorea victoria*, and was first described in 1962 [19]. GFP emits bright green light when simply exposed to ultraviolet (UV) or blue light, unlike other bioluminescent reporters. The emission of green light is due to the transfer of energy from the photoprotein, aequorin, to GFP [20]. The cDNA of GFP was cloned from *Aequorea victoria* in 1992 [21]. GFP is a 238-amino acid protein with a molecular weight of 27 kDa [18]. GFP has a major absorption peak at 395 nm and a minor peak at 470 nm with a single emission peak at 509 nm [18]. GFP has several advantages, such as species-independent fluorescence and, unlike *lacZ*, it requires no substrate, cofactor or additional proteins for detection. Unlike other reporters, e.g., luciferase or fluorescence-tagged antibodies, GFP does not necessarily require pretreatment prior to detection, such as fixation techniques that are toxic to the cells under investigation [22]. As such, GFP has been widely used. Multiple reviews of GFP and its variants have appeared [16, 23–25] (*see* **Note 1**).

The BEVS has been increasingly utilized for a variety of purposes, ranging from gene therapy [26] to vaccine development [27, 28], and also importantly as one of the primary platforms for

recombinant protein production [29]. That is, the BEVS is both effective and convenient for the overproduction of recombinant proteins in eukaryotic cells [30–34]. The strong polyhedrin (polh) promoter [35, 36] and the 10 kDa fibrous polypeptide (p10) promoter [33, 37], are both active in the very late phase of virus infection and when used to drive heterologous protein production can result in the accumulation of over 50 % of the total protein [38]. Also, relatively recent metabolic engineering approaches to increase yield further have appeared, such as those using RNAi [39–41]. Although high protein productivity can be achieved with the use of late promoters (polh or p10), these might not be ideal for probing the infection process due to the lag time between infection and polh-mediated protein synthesis (48 h post-infection). Early on, Crawford and Miller [42] characterized the role of several early baculovirus genes on the expression of late viral genes and noted that the early-to-late (ETL) promoter might advantageous for aiding the identification of occlusion-body negative recombinants via expression of β-galactosidase. Similarly, Richardson et al. [13] demonstrated the use of early and late promoters, including the ETL promoter, to facilitate the plaque assay. Hence, an optimal system might utilize the ETL promoter for monitoring infection [43, 44] and the p10 or polh promoters for protein production.

## 2 Materials

### 2.1 Cell Culture and Medium

1. Insect cells (*Spodoptera frugiperda*, Sf-9) are obtained from American Type Culture Collection (ATCC, CRL 1711).
2. TNM-FH insect medium supplemented with 10 % fetal bovine serum or SF900-II serum-free medium (Life Technologies).
3. 0.4 % trypan blue solution.
4. Hemacytometer.
5. 60 mm tissue-culture dish.
6. 25 $cm^2$ tissue-culture flask.

### 2.2 Expression Vector and Baculovirus

1. The vector pGFPuv (Clontech).
2. The transfer vector pVL1392 (BD Biosciences).
3. The transfer vector pBlueBacHis2/CAT (Life Technologies).
4. The recombinant transfer vector pVLGFPuv, obtained from pVL1392 and pGFPuv [45].
5. The recombinant transfer vector pBBH(Δ*lacZ*)GFPuv, obtained from pBlueBacHis2/CAT and pGFPuv [44].
6. The recombinant transfer vector pBBH-GFPuv/CAT, obtained from pBlueBacHis2/CAT and pGFPuv [44].

7. Linearized BaculoGold™ Wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) DNA (BD Biosciences).
8. Bac-N-Blue™ wild-type AcMNPV DNA (Life Technologies).
9. The recombinant baculovirus, *v*GFPuv, obtained by co-transfecting Sf-9 cells with pVLGFPuv and linearized BaculoGold™ wild-type AcMNPV DNA.
10. The recombinant baculovirus, *v*$P_{ETL}$-GFPuv, obtained by co-transfecting Sf-9 cells with pBBH(Δ*lacZ*)GFPuv and Bac-N-Blue™ wild-type AcMNPV DNA.
11. The recombinant baculovirus, *v*PH-GFPuv/CAT, obtained by co-transfecting Sf-9 cells with pBBH-GFPuv/CAT and Bac-N-Blue™ wild-type AcMNPV DNA.
12. Phosphate buffered saline (PBS) buffer.
13. UV transilluminator.
14. 96-well plate.
15. Fluorescence spectrometer.
16. Fluorescent microscope.

### 2.3 Western Blotting for GFP

1. Sample buffer (5×): 125 mM Tris–HCl, pH 6.8, 10 % (w/v) glycerol, 10 % (w/v) SDS, 5 % (w/v) β-mercaptoethanol, and 0.25 % (w/v) bromophenol blue. Store at room temperature.
2. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8, 10 % (w/v) SDS. Store at 4 °C.
3. Running buffer (4×): 1.5 M Tris–HCl, pH 8.8, 10 % (w/v) SDS. Store at 4 °C.
4. 2.5 % (v/v) Triton X-100: 25 mL Triton X-100, add distilled water to 1 L. Stir overnight to dissolve, autoclaving is not necessary.
5. 30 % (w/v) acrylamide–bis (37.5:1) solution (*see* **Note 2**).
6. *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED).
7. 10 % (w/v) ammonium persulfate: prepare solution in water and immediately freeze in single use (200 μL) aliquots at −20 °C.
8. Water-saturated isobutanol: shake equal volumes of water and isobutanol in a glass bottle and allow for separation. Use the top layer. Store at room temperature.
9. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad). Load 10 μL/well for mini-gels.
10. Coomassie blue stain: 1.625 g Coomassie blue, 450 mL distilled water, 450 mL methanol and 100 mL glacial acetic acid. Store at room temperature.
11. Coomassie blue destain: 450 mL distilled water, 450 mL methanol and 100 mL glacial acetic acid. Store at room temperature.

12. Bjerrum and Schafer-Nielsen transfer buffer: 48 mM Tris, 39 mM glycine, 20 % (v/v) methanol, pH 9.2 (Do not adjust the pH of this buffer).
13. Tris-buffered saline (TBS): 20 mM Tris–HCl, 500 mM NaCl, pH 7.5. Adjust pH with concentrated HCl.
14. TBS with Tween (TTBS): 0.5 mL Tween 20 to 1 L of TBS.
15. Blocking buffer: 5 % (w/v) nonfat dry milk in TTBS.
16. Primary antibody dilution buffer: TTBS supplemented with 2 % (w/v) fraction V bovine serum albumen (BSA).
17. Primary antibody: Polyclonal anti-rGFP antibody (Clontech).
18. Secondary antibody: 1:5000 dilution goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories).
19. BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) color development reagent.
20. Supported nitrocellulose membrane.
21. 3 MM chromatography paper.

## 3 Methods

### 3.1 Construction of Recombinant Expression Vectors and Establishment of Recombinant Baculoviruses

1. Excise the *gfpuv* gene from the pGFPuv plasmid using *Pst*I and *Eco*RI digestion.
2. Insert the *gfpuv* gene (from **step 1**) under the polyhedron (polh) promoter of the pVL1392 baculovirus transfer vector to obtain the pVLGFPuv transfer vector [45], which is subsequently amplified in *E. coli* (*see* **Note 3**).
3. Co-transfect $2 \times 10^6$ Sf-9 cells with the 5 μg pVLGFPuv transfer vector (from **step 2**) and 0.5 μg linearized BaculoGold™ wild-type AcMNPV DNA to obtain the *v*GFPuv recombinant baculovirus (*see* **Note 4**).
4. Construct pBlueBacHis2(Δ*lacZ*) transfer vector [44] by polymerase chain reaction (PCR) amplification around the *lacZ* gene sequence of the pBlueBacHis2/CAT transfer vector using the primer sets (5′-gctctagattttagcagtgattctaattgcagc-3′ and 5′-ccgcggccgccaccatacgcccgaaaccagtac-3′, 5′-gggcggccgccactctcagtacaatctgctctg-3′ and 5′-gctctagacggccgctgaggccgatactgtcgtcgtc-3′).
5. Excise the *gfpuv* gene from the pGFPuv plasmid using *Eag*I and *Xba*I digestion.
6. Insert the *gfpuv* gene (from **step 5**) in frame under the ETL promoter of the pBlueBacHis2(Δ*lacZ*) transfer vector (from **step 4**) resulting in a transfer vector denoted pBBH(Δ*lacZ*) GFPuv.

7. Co-transfect $2 \times 10^6$ Sf-9 cells with the 5 μg pBBH(Δ*lacZ*) GFPuv transfer vector (from **step 6**) and 0.5 μg Bac-N-Blue™ wild-type AcMNPV DNA to obtain the $v\mathrm{P}_{\mathrm{ETL}}$-GFPuv recombinant baculovirus.
8. Amplify the *gfpuv* gene from the pGFPuv plasmid using the PCR (primers: 5′-ggctagcatgagtaaaggagaagaacttttc-3′ and 5′-ggctagctttgtagagctcatccatgcc-3′).
9. Digest the PCR-amplified *gfpuv* gene using *Nhe*I.
10. Insert the *gfpuv* gene (from **step 9**) in frame under the polh promoter of the pBlueBacHis2/CAT transfer vector resulting in a fusion transfer vector denoted pBBH-GFPuv/CAT [44] for expression of fusion foreign protein, GFP and chloramphenicol acetyl-transferase (CAT).
11. Co-transfect $2 \times 10^6$ Sf-9 cells with the 5 μg pBBH-GFPuv/CAT transfer vector (from **step 10**) and 0.5 μg Bac-N-Blue™ wild-type AcMNPV DNA to obtain the *v*PH-GFPuv/CAT recombinant baculovirus.
12. Propagate recombinant virus stocks in Sf-9 cells using Hink's TNM-FH insect medium supplemented with 10 % fetal bovine serum in 25 $cm^2$ tissue-culture flasks at 27 °C.
13. Grow Sf-9 cells in SF900-II SFM medium and subculture routinely every 3–4 days [1].
14. Count cell number using a hemacytometer.
15. Determine cell viability by trypan blue exclusion using a 0.4 % (w/v) solution.

### 3.2 End-Point Dilution and Titer Determination Using GFP

This method is a modification of the end-point dilution method (*see* Chapter 10) and is facilitated by incorporating a GFP marker for determining the titer of a virus stock (*see* **Note 5**). Since GFP imposes minimal metabolic burden on the host cells, it is anticipated that the use of GFPuv as a fusion partner or co-expressed with a desired foreign protein (under different promoters) will facilitate recombinant baculovirus titering in a non-intrusive manner.

1. Dilute the virus stock samples serially $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ by transferring 100 μL from the previous diluted sample into a new tube that has 900 μL fresh medium, e.g., 100 μL of the $10^{-4}$ sample into 900 μL fresh medium to obtain the $10^{-5}$ sample. Each sample has an equal final volume of 900 μL, except the $10^{-8}$ dilution, which has a final volume of 1000 μL.
2. Harvest $3 \times 10^6$ Sf-9 cells from the exponential growth phase and add fresh medium to obtain a total volume of 30 mL. This will result in a cell density of $10^5$ cells/mL.
3. Mix 0.5 mL of each virus dilution (**step 1**) with 4.5 mL of the Sf-9 cell suspension (**step 2**) to obtain a total volume of 5.0 mL. Gently mix.

4. Pipet 100 µL of the virus/cell samples obtained in **step 3** into 12 replicate wells of a 96-well plate for each virus dilution. Prepare a total of four plates for time course observation (*see* **Note 6**).
5. Incubate the plates obtained in **step 4** at 27 °C for 4–7 days.
6. Check each well for virus infection on a UV table. Green light, which is due to GFP expression, indicates virus infection and thus a positive well (*see* **Note 7**).
7. Calculate the titer (pfu/mL) of the recombinant baculovirus *v*GFPuv using the standard method described in Chapter 10 (*see* **Note 8**).

### 3.3 Early Monitoring of Viral Infection Using ETL-GFP Strategy

This method combines the advantages of the ETL promoter and GFP for a simpler and earlier monitoring of baculovirus infection.

1. Infect Sf-9 cells during exponential growth (~$10^6$ cells/mL) with a predetermined volume of the *v*$P_{ETL}$-GFPuv and *v*PH-GFPuv/CAT recombinant baculoviruses to yield a multiplicity of infection (MOI) of 5 (*see* **Notes 9** and **10** and Chapter 1).
2. Collect culture samples initially every 6 h for the first 60 h post-infection (p.i.) and then every 12 h thereafter.
3. Prepare whole cells (from **step 2**) without medium by centrifugation and resuspend in PBS buffer.
4. Quantify GFP using whole cell fractions in PBS buffer (from **step 3**) by fluorescence spectrometry at an excitation wavelength of 395 nm and an emission wavelength of 509 nm.
5. Examine GFP production (from whole cell fractions) via Western blot as a function of time post-infection using samples collected in **step 2**.
6. Prepare a 1.5-mm thick, 10 % gel by mixing 7.5 mL of 4× separating buffer, with 10 mL acrylamide/bis solution, 12.5 mL water, 100 µL ammonium persulfate solution, and 20 µL TEMED. Pour the gel, leaving space for a stacking gel, and overlay with water-saturated isobutanol. The gel should polymerize in about 30 min.
7. Pour off the isobutanol and rinse the top of the gel twice with water.
8. Prepare the stacking gel by mixing 2.5 mL of 4× stacking buffer with 1.3 mL acrylamide/bis solution, 6.1 mL water, 50 µL ammonium persulfate solution, and 10 µL TEMED. Use about 0.5 mL of this to quickly rinse the top of the gel and then pour the stack and insert the comb. The stacking gel should polymerize within 30 min.
9. Prepare the running buffer by diluting 100 mL of the 4× running buffer with 400 mL water in a measuring cylinder. Cover with Para-Film and invert to mix.

10. Once the stacking gel has set, carefully remove the comb and use a 3-mL syringe fitted with a 22-gauge needle to wash the wells with running buffer.
11. Add the running buffer to the upper and lower chambers of the gel unit and load 50 μL of each sample in a well. Include one well for prestained molecular weight markers.
12. Complete the assembly of the gel unit and connect to a power supply. The gel can be run either overnight at 50 V or, if cooling is available for the gel unit, then during the day (about 5 h) at 20 mA through the stacking gel and 30–40 mA through the separating gel.
13. Electrophoretically transfer the samples that have been separated by SDS-PAGE onto supported nitrocellulose membranes (*see* **Note 11**).
14. Incubate the nitrocellulose in 50 mL blocking buffer for 1 h at room temperature on a rocking platform.
15. Discard the blocking buffer and rinse the membrane quickly prior to addition of a 1:1000 dilution of the anti-GFP antibody in TTBS/2 % BSA for 1 h at room temperature on a rocking platform.
16. Remove the primary antibody and wash the membrane three times for 5 min each with 50 mL TTBS.
17. Prepare fresh secondary antibody for each experiment as 1:5000-fold dilution in blocking buffer and add to the membrane for 30 min at room temperature on a rocking platform.
18. Discard the secondary antibody and wash the membrane six times for 10 min each with TTBS.
19. During the final wash, warm 2 mL aliquots of each portion of the BCIP/NBT reagent separately to room temperature.
20. After removing final wash from the blot, add the BCIP/NBT reagent to the blot.
21. The examples of the Western blots and time courses are shown in Fig. 1 (*see* **Note 12**) and Fig. 2 (*see* **Note 13**), respectively.

## 4 Notes

1. In this research, a GFP variant, GFPuv, which is optimized for UV excitation [22], can be used. GFPuv is 18 times brighter than wild-type GFP and can be easily detected by the naked eye when excited with standard, long-wave UV light (e.g., source for many DNA transilluminator light tables). Note that this variant is ideally suited for this work because there is a large difference between the optimal excitation and emission wavelengths and because its intrinsic brightness is so high.

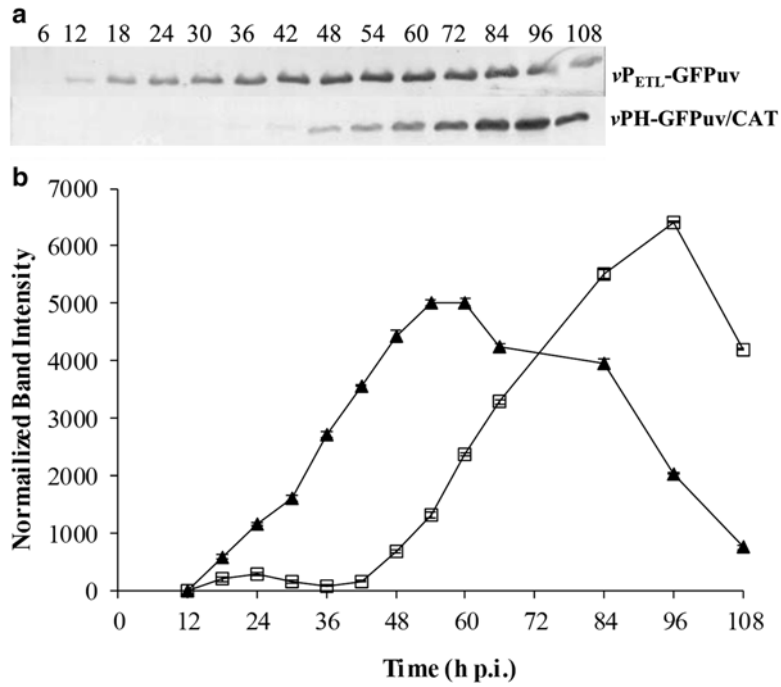


**Fig. 1** Western blot analyses of GFP expression under ETL (*filled triangle*) and polh (*open square*) promoters. (**a**) Developed Western blots. (**b**) Quantified results that were normalized to the first non-zero intensities from scanned images (12 h p.i., ETL signal)

Also, other GFP variants and fluorescent proteins, e.g., EGFP, BFP, YFP, and CFP [16, 23–25], could be used depending on the specific purposes or situations (e.g., *see* Luo et al. [46] and Yi et al. [47]).

2. This is a neurotoxin when unpolymerized and so care should be taken to avoid exposure.
3. This strategy leaves the p10 promoter (another strong baculovirus promoter) available for the expression of genes of interest from the same virus.
4. Successful co-transfection can be observed through green fluorescence using a fluorescence microscope.
5. This method has several advantages. First, this modified method is substantially faster than the normal end-point method. This is an important advantage because we currently wait longer (about 5–7 days for end-point dilution and 6–7 days for plaque assays) for detecting infection. Second, this method makes unambiguous the determination of positive (infected) wells. That is, the titer of recombinant baculovirus is obtained by simply placing the 96-well plate on the UV box and counting the green light emitting wells.

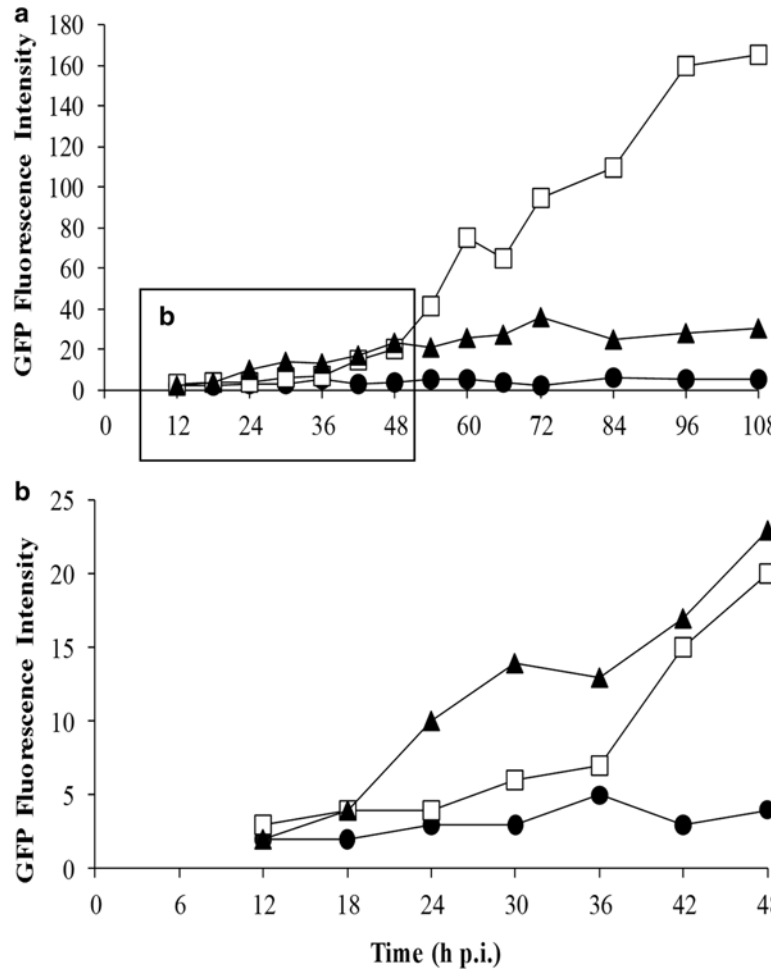


**Fig. 2** GFP fluorescence intensity for control (*filled circle*) and cultures infected with $vP_{ETL}$-GFPuv (*filled triangle*) and *v*PH-GFPuv/CAT (*open square*). (**a**) Fluorescence intensity over entire culture time. (**b**) Fluorescence intensity for first 48 h p.i.

Note that the GFPuv marker can also be applied to plaque assays for more easily visualizing plaques.

6. Four plates are used so that results can be checked daily from day 4 to day 7, in the event that UV lights are to damage the cells and a given plate can only be read once. Although we have hypothesized that UV light may have a damaging effect on the cells, we have not experienced such difficulty, particularly because exposure time is routinely kept brief.
7. Furthermore, we can check the plates for infection each day during the 7-day incubation to test whether the results are dependent on incubation time.
8. We can check the infected wells by eye and count green light-emitting wells. The orders of magnitude of calculated titer are

the same ($10^7$) in all end-point dilution plates for each day. Furthermore, the values are similar among all samples and within the standard error of the calculated average titer, $5 \times 10^7$ pfu/mL. Finally, there is no significant difference observed in plates read once and plates read three times (on consecutive days) after illumination on the UV table.

9. The intent of this experiment is to compare the infection of these two recombinant baculoviruses, i.e., to compare the use of the ETL promoter to that of the polyhedrin promoter.
10. Effectiveness of baculovirus infection depends on cell culture conditions such as virus titer, initial cell number, initial cell viability, and medium composition.
11. The colored molecular weight markers should be clearly visible on the membrane.
12. Western blots indicate a clear shift of ~18 h for ETL-driven expression in comparison to that expressed via the polh promoter. The ETL-driven GFP reaches a maximum at 50 h p.i., after which the accumulation level declines steadily. The decrease is possibly due to proteolysis [48] and/or cell lysis following virus infection, although the latter is less likely given the previously observed persistence of GFP from the very late polh vectors. Under the polh promoter, GFP is detected at approximately 42 h p.i. with a maximum at 96 h p.i. followed by a sharp decrease. These results correspond well to the expected profile, where immediate-early and delayed-early genes are expressed between 0 and 10 h p.i., the transition to the late phase occurring between 10 and 15 h p.i., and the very late genes express after 18 h p.i. [49].
13. Consistent with Western blots, the GFP fluorescence intensity under the polh promoter increases rapidly after 42 h p.i. until reaching a maximum at ~96 h p.i., but without a marked decline. Under the early ETL promoter, GFP fluorescence remains low and fairly constant after 48 h p.i., but increases initially near 18 h p.i. (*see* inset in Fig. 2a) resulting in a similar 18 h time shift observed by Western analysis.

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# Chapter 23

# Tubular Bioreactor for Probing Baculovirus Infection and Protein Production

**Hsuan-Chen Wu, Yu-Chen Hu, and William E. Bentley**

## Abstract

Probing the baculovirus infection process is essential in optimizing recombinant protein production. Typically, researchers monitor the infection process in stirred reactors that contain cells that have been infected at different times after virus inoculation, particularly if cells pass the primary infection and become infected by progeny virus. This chapter describes several alternative bioreactor systems for baculovirus infection. We provide an example alternative system that holds promise to avoid asynchronous distributions in infection time. Namely, we describe a two-stage reactor system consisting of an upstream continuous stirred tank reactor and a downstream tubular reactor with segmented plug flow for probing baculovirus infection and production.

**Key words** Tubular reactor, Baculovirus infection, Insect cell culture, Monitoring

## 1 Introduction

The baculovirus expression vector system (BEVS) (which utilizes host insect or, recently, mammalian cells) has been widely used for recombinant protein production [1–4]. To cultivate cells and produce proteins of interest, a variety of stirred tank bioreactors are commercially available as well as some novel reactor systems, e.g., TubeSpin reactors [5], packed-bed reactors [6–8], insect larvae [9, 10], hollow fiber reactors [11], Wave™ bioreactors (*see* Chapter 12), and continuous stirred tank reactors (CSTR) [12–15]. However, due to the eventual host lysis, virus genome instability, and the intrinsic heterogeneous infection process of the baculovirus system [16], designing and maintaining optimal conditions has been difficult to achieve or sustain for long processing times. In order to characterize the BEVS, simplified models relating the multiplicity of infection (MOI; effectively, the infectious virus particle–cell ratio) to the product yield have been proposed [17–21], but it is generally difficult to interpret these models that segregate the various cell states in a way that accurately and simply indicates

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the current state of the infection process. This difficulty, in part, stems from the population distribution of cells infected at different times following virus inoculation within the stirred tank or batch reactor. For example, after virus addition to the stirred tank reactor, some cells may be infected immediately while other cells are infected hours later. The proportion of cells that are not immediately infected gets higher as the initial MOI is reduced below ~5. The analysis of cells made at any particular time post-infection, therefore, actually represents a distribution of cells infected over a historical period of time during the infection process; without recognizing this one might misinterpret the results. To circumvent this problem, a two-stage bioreactor system was reported consisting of an upstream CSTR that provides uninfected cells, with constant viable cell density, continuously to a downstream tubular reactor with segmented plug flow for virus infection [22]. The liquid volume within the segment is small so that nearly all cells in the same liquid element are infected synchronously and have nearly identical residence times (i.e., the time required by the liquid element to move from the entrance to the exit of the tubular reactor). Thus, a homogeneous infection can be accomplished and tracked. Also by taking samples in the liquid segment at different distances down the tubular reactor (i.e., different times post-infection), one can monitor the infection kinetics and subsequent metabolism and physiology of infected insect cells.

## 2 Materials

### 2.1 Cell Culture, Medium, and Assays

1. Insect cells (*Spodoptera frugiperda*, Sf-9) are obtained from American Type Culture Collection (ATCC, CRL 1711).
2. Ex-Cell™ 401 serum-free media (Sigma) is supplemented with 0.1 (w/v)% Pluronic® F-68, 100 mg/L ampicillin (*see* **Note 1**), 2.5 g/mL Fungizone, and 0.02 % antifoam (Dow Corning 10 % FG-10 antifoam emulsion, food grade; Corning).
3. Hemacytometer and trypan blue for cell count and viability determination.
4. Phosphate buffered-saline (PBS; pH 7.4): 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM $KH_2PO_4$.
5. 25 $cm^2$ tissue culture treated flasks (T-25 flasks; Corning) are used for cell cultivation.
6. Recombinant baculovirus expressing the reporter protein. In this case, vIBD-7 [23], a recombinant baculovirus expressing β-galactosidase (β-gal), is used.
7. O-nitrophenyl-β-D-galactopyranoside (ONPG) for β-gal activity assay.

### 2.2 Two-Stage Bioreactor System

1. Culture room controlled at 27–28 °C.
2. A glass-blown jacketed spinner flask (working volume = 200 mL) made in house.
3. One-liter medium bottles with multi-port cap assemblies (Bellco Biotechnology) to serve as medium reservoir and waste jar.
4. Stirrer plate.
5. Water bath.
6. Needles (22-gauge) and syringe (1 mL) for sampling.
7. Rubber stopper (No. 10).
8. Stirrer assembly (Bellco Biotechnology).
9. Masterflex precision L/S peristaltic pumps (Cole Palmer).
10. Microprocessor-controlled peristaltic multichannel pump (Watson Marlow, model 505Du) and a 4-channel pump head (model 205BA).
11. Watson-Marlow silicone tubing (ID = 1.14 mm) and MasterFlex no. 14 tubing (length = 7.6 m, ID = 1.6 mm).

## 3 Methods

1. The bioreactor system is set up as shown in Fig. 1 and is operated in a laminar flow hood located in a culture room controlled at 27–28 °C (*see* **Note 2**).
2. The water bath and pump 3 are turned on for temperature control and aeration, respectively. The suspended cells (200 mL) are inoculated into the stirred tank bioreactor at $3 \times 10^5$ cells/mL and operated in the batch mode (agitation speed = 80 rpm).
3. When the cells enter the exponential phase (~$7–7.5 \times 10^5$ cells/mL, *see* **Note 3** and Chapter 1), the bioreactor operation is switched to the continuous mode. Pumps 1 and 2 are turned on to withdraw the spent medium and supply fresh medium continuously, thereby keeping the medium level in the bioreactor constant. The CSTR is operated at a dilution rate (volumetric flow rate of medium divided by working volume) of 0.0315 $h^{-1}$.
4. Samples are withdrawn daily from the CSTR via the sampling port for measuring cell density to determine when steady state (that is, when the cell density remains constant as a function of time) is reached.
5. After steady state is reached in the CSTR, turn on the multichannel pump 4. The operation of pump 4 withdraws cells from the CSTR, enabling the subsequent mixing of cells with virus solution and fresh medium, as well as introduction of air.

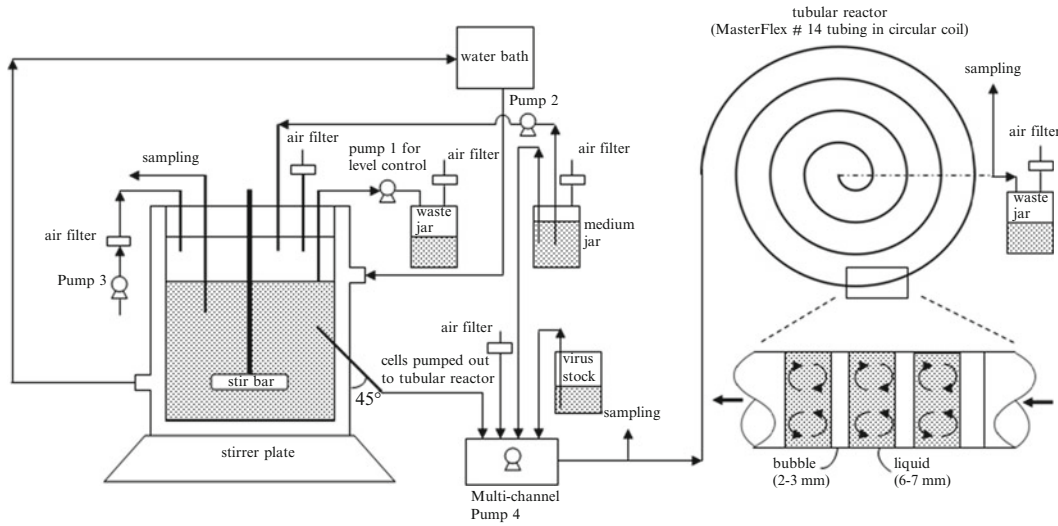


**Fig. 1** Schematic diagram of the CSTR-tubular two-stage bioreactor system. The CSTR is composed of a glass-blown jacketed spinner flask (working volume = 200 mL) with a medium reservoir, a waste jar and pumps for medium and gas flow. The headplate consists of a rubber stopper (No. 10) drilled with six holes for stirring, venting, sampling, aeration, medium inlet, and level control. The rotation of stirrer bar is driven by a stirrer plate. The CSTR temperature is maintained at 28 °C using a circulating water bath. A thin glass tube (≈2 mm ID) is annealed to the CSTR and protrudes from the side of the reactor at a 45° angle downward from the vertical, and is connected via Watson-Marlow silicone tubing (ID = 1.14 mm) to the tubular reactor entrance, thereby allowing for the cells to flow to the tubular reactor. The tubular reactor is comprised of a multichannel pump (Watson Marlow, model 505Du) and a semipermeable silicone tube (MasterFlex no. 14, length = 7.6 m, ID = 1.6 mm). The pump accommodates a 4-channel pump head (model 205BA) and can be operated as slowly as 0.2 rpm for transporting cells, virus solution, fresh medium and air. The long tube is coiled horizontally as a series of concentric circles. Virus solution is introduced to the tubular reactor at the entrance. Watson-Marlow silicone tubing (ID = 1.14 mm) is used for precise pumping of air into the tubular reactor through a Y-junction in the inlet line. The expanded panel of the tubular reactor illustrates the bubble/liquid flow pattern and the expected velocity profile in the liquid segment

The mixing creates a segmented flow pattern (Fig. 1, *see* **Note 4**) whose validity has been demonstrated previously [22].

6. Twenty two-gauge needles connected to syringes are pierced through the entrance and exit of the tubular reactor for sampling.
7. Pull the syringe to carefully take samples. When taking samples, pump 4 is shut down temporarily and the tubular reactor is clamped at the upstream and downstream of the sampling point. After sampling, the reactor is de-clamped and then pump 4 is turned on again. The infected cells are washed with PBS, resuspended in fresh medium, and incubated in a T-25 flask at 28 °C.
8. After the linear velocity of the segmented liquid becomes steady, repeat the sampling process (*see* **Note 5**).

9. The infected cells are incubated for 72, 96, 120, 144, 168, and 192 h and then the reporter protein activities are measured. In this case, vIBD-7 is used as the recombinant baculovirus; thus, β-gal activities are measured [24]. If recombinant baculovirus expressing other reporter protein (e.g., green fluorescent protein, *see* Chapter 22) is used, then the corresponding assays may be performed.
10. Adjust the speed of pump 4 so as to change the linear velocity and the corresponding residence times (RTs) of the liquid element within the tubular reactor (*see* **Note 6**). Repeat **steps 7–9**.
11. The completeness of virus infection can be monitored from the time course curves of β-gal expression at different RTs. Figure 2 shows a lower β-gal yield at RT = 1.6 h (MOI = 60, *see* **Note 7**), indicating that the infection is incomplete. At higher RTs (3.2 and 5.1 h), there are no significant differences in either the rate of increase or the level of β-gal activity, indicating that at MOI = 60 the infection process is completed prior to 3.2 h post-infection (*see* **Note 8**).

## 4 Notes

1. Ampicillin is used in lieu of gentamycin because gentamycin interferes with antifoam, thereby forming gel-like substances.
2. No additional temperature control is required for the tubular bioreactor since insect cells grow optimally at 27–28 °C. Furthermore, the pH is not controlled since the insect cell medium has a strong buffer capacity and does not change significantly under these operating conditions.

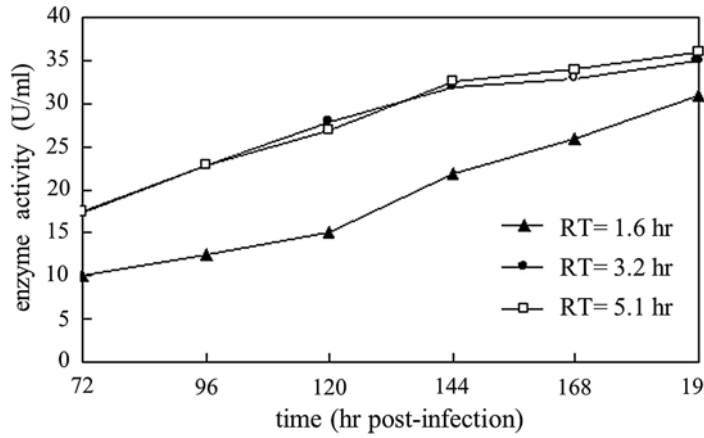


**Fig. 2** Time-course profile of β-gal activity in cells infected for different residence times (RTs) at MOI = 60. Cells are collected at the exit of the tubular reactor operated at different RTs, washed in PBS and seeded to T-25 flasks for expression

3. Since the CSTR is only for continuous cell supply, the cell density may be changed as long as the cells remain in the exponential growth phase.
4. It is critical that the multichannel pump (Watson Marlow) provides constant and stable liquid flow so as to generate uniform segmented liquid elements.
5. The needles are pierced through the tubular reactor once. For subsequent sampling, change the syringe only.
6. The adjustment of the multichannel pump speed changes the liquid flow rate and linear moving velocity of the liquid elements, which subsequently changes the residence time of the liquid element. The segmented flow pattern eliminates the residence time distribution common in CSTR and hence minimizes the infection time distribution. Therefore, by operating at different residence times one might be able to track cell physiology and metabolism during the post-infection period without interference from population distributions by taking samples at the exit of the reactor.
7. The MOI can be controlled by varying the flow rate ratio of cells and virus stock solution. To study the infection process at different MOI's, adjust the concentration (pfu/mL) of the virus stock solution or vary the size of the tubing for transporting virus solution; alternatively, change the cell density exiting from the CSTR.
8. One problem associated with this system is that at low flow rates, cells may settle between the exit of the CSTR and the entrance of the tubular reactor due to the lack of the bubbles (bubbles in the tubular reactor effectively prevent the cell settling). Some possible solutions to the settling problem include the introduction of air bubbles in the CSTR exit, increasing mixing by an inline static mixer between the CSTR exit and the tubular reactor inlet pump, and adding methylcellulose or dextran sulfate to alleviate cell aggregation.

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# Chapter 24

# Gene Silencing in Insect Cells Using RNAi

**Hsuan-Chen Wu, John C. March, and William E. Bentley**

## Abstract

A technique is described for synthesizing and transfecting double stranded RNA (dsRNA) for RNA interference (RNAi) in Sf-21 cell culture. Transfection with dsRNA only requires an hour and the cells usually recover within 12 h. Suggestions for designing dsRNA are included in the methods. Furthermore, websites are provided for rapid and effective dsRNA design. Three kits are essential for using the described methods: RNAqueous®-4PCR, Megascript™ T7 kit, and the Superscript™ III kit from Life Technologies, Inc.

**Key words** RNAi, RNA interference, Gene silencing, dsRNA, mRNA, Transcription, T7, SP6

## 1 Introduction

Gene silencing using RNA interference (RNAi) allows for the transient and, more recently, stable silencing of genes without the difficulty of making knockout cell lines [1–6]. Furthermore, in the case of transient silencing, the phenotype associated with the return of gene function can be observed. RNAi techniques for insect cell culture first were demonstrated in *Drosophila* S2 cells. Since then, some Lepidopteran lines have been reported to be sensitive to RNAi, including Sf-9, Sf-21, *Bombyx mori*, and *Trichoplusia ni* [2–5, 7–10]. One of the most commonly used and well established strategies for silencing genes in insect cell culture focuses on in vitro-synthesized double stranded RNA (dsRNA). In these studies, dsRNA is synthesized enzymatically in vitro and then transfected into the cells [4, 11]. RNAi silencing of both viral [12–14] and host [1, 2, 15] genes has been demonstrated. The use of RNAi allows for transient inhibition of several genes that are expected to increase titer or product yield [1–3, 12–14]. More recently, it has been shown that baculovirus can be used to deliver microRNA or short interfering RNA (siRNA) to a host for in vivo transcription and silencing [16, 17] (*see* **Note 1**). The method described here is a modified version [18] for in vitro dsRNA synthesis and transfection into Sf cells [9, 11].

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## 2 Materials

### 2.1 Designing dsRNA

The selection of a silencing target should be based on sound a priori knowledge of the pathways collateral to the system under study. Unintentional manipulation of essential processes can hinder product yield. Therefore, the more well-characterized a potential target is, the greater the likelihood of success. There are several venues available for designing dsRNA. One is website-based software such as that published by Arizman and coworkers that allows for optimized design and provides access to published predesigned dsRNAs [19]. The key to the author's approach is an algorithm for determining off-target effects for some organisms, including *Drosophila melanogaster*. Another website for off-target effects is provided by Naito et al. [20]. It is highly recommended that off-target effects are considered in designing dsRNA for gene silencing, since even the smallest effective dsRNA (~300 bp) can have 14 small, 21 bp sequences (after digestion with Dicer) that can potentially silence a homologous transcript.

Design of dsRNA requires the sequence of the target gene. These can be found on Silkbase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) for *B. mori*, Flybase (http://flybase.org/) for *Drosophila*, SPODObase (http://bioweb.ensam.inra.fr/spodobase/) for *Spodoptera frugiperda* or ButterflyBase (http://www.butterflybase.org) for butterflies and moths. Also a major international effort is underway to obtain sequences for other lepidopteran cell lines (http://papilio.ab.a.u-tokyo.ac.jp/lep-genome/index.html). After the sequence is obtained, design can be completed relatively quickly at one of the aforementioned websites. Alternatively, the Drosophila RNAi Screening Center (DRSC; http://www.flyrnai.org) provides the library of dsRNAs for directly targeting known and predicted genes in *Drosophila*.

### 2.2 Making the DNA Template

1. RNA extraction kit. Commercial column-based purification kits are often used for total RNA extractions; typically using affinity chromatography. Depending on the conditions, these are more rapid and produce RNA of higher quality when compared to conventional phenol–chloroform extraction procedures. The column-based RNA extraction kits are widely available from a number of manufacturers. For the demonstration included here, we use the protocol of RNAqueous®-4PCR kit from Life Technologies (Grand Island, NY); it serves as an example of the many available purification systems. This kit includes:
   (a) Lysis/binding buffer. This buffer contains β-mercaptoethanol, sodium orthovanadate, and a proprietary mixture intended to inhibit RNAases, degrade DNA, and protect RNA.

(b) Columns for immobilizing RNA.

(c) Washing buffers. There are two washing buffers. The first wash buffer contains guanidinium thiocyanate, a potentially hazardous material. The final wash buffer contains 80 % ethanol.

(d) DNAase 1.

(e) DNAase inactivation reagent. This polymeric emulsion binds to the DNAase 1 and removes it from solution.

(f) Elution buffer. This buffer contains 0.1 mM EDTA.

2. Nuclease-free water. This is available from Life Technologies in up to 1 L bottles, and is provided free with the RNAqueous®-4PCR kit.

3. Reverse transcriptase kit (for making cDNA from RNA). Reverse transcriptase kits are available from a number of sources. The protocol described here makes use of the Superscript™ III RNA-dependent DNA polymerase from Life Technologies. This Superscript™ III kit includes:

(a) Deoxynucleotides (dATP, dCTP, dGTP, dTTP).

(b) DTT (reducing agent).

(c) $MgCl_2$.

(d) OligodT primers.

(e) 10× reaction buffer.

(f) RNAase inhibitor.

(g) Superscript™ III RNA-dependent DNA polymerase.

(h) RNAase H and RNAase H buffer.

4. 10 μM primer working stocks. Primers to amplify the section of gene that has been selected as a target should be diluted to 10 μM. There should be two sets of primers: one set (forward and reverse) for PCR amplification of the template, and another set (forward and reverse) to add the T7 RNA polymerase site (5′-TAA TAC GAC TCA CTA TAG GG-3′) to the template.

5. Vent® DNA polymerase and Thermopol buffer (New England Biolabs (NEB)). The buffer is supplied with the enzyme.

6. 10 mM deoxynucleotide mix (NEB). The mix includes 10 mM each of dATP, dCTP, dGTP, and dTTP.

7. 50 mM $MgCl_2$ solution. This solution usually comes with the DNA polymerase to increase the $MgCl_2$ concentration when the PCR result is unsatisfactory.

### 2.3 In Vitro dsRNA Synthesis

1. In vitro transcription kit. Many in vitro RNA synthesis kits are commercially available; most originated from bacteriophage studies. Several varieties of RNA polymerase expression systems, including T7, SP6 or T3, are developed based on their

unique promoter sequences. For the current demonstration, we selected one of the most popular methods that exploit RNA polymerase, T7. The following list of materials is from a Megascript™ kit (Life Technologies):

(a) 80 μL enzyme mix (T7 RNA polymerase).

(b) 80 μL 10× reaction buffer: salts (proprietary), buffer, dithiothreitol, and other ingredients.

(c) 80 μL of each 75 mM nucleotide solution (ATP, CTP, GTP, UTP).

(d) 45 μL of DNAase 1, 2 U/μL (RNase-free).

(e) 1 mL ammonium acetate stop solution.

(f) 1 mL nuclease-free water.

(g) Gel loading buffer: 95 % formamide, 0.025 % xylene cyanol, 0.025 % bromophenol blue, 18 mM EDTA, 0.025 % SDS.

2. Phenol–chloroform solution. Use a 1:1 mix of buffer saturated phenol–chloroform. Buffer-saturated phenol–chloroform has been saturated with Tris–HCl to hold a pH of 7.4 and remain liquid at room temperature. It is commercially available (store at 4 °C).
3. Isopropanol (store at –20 °C).
4. Ethanol (store at –20 °C).
5. Nuclease-free pipet tips and microcentrifuge tubes.
6. Vacuum for drying the dsRNA pellet.
7. Water bath at 65 °C.
8. DNase 1 and RNAase H from previous steps.

### 2.4 Transfecting Sf-21 Cells in Culture

1. Sf900 II serum-free medium (SFM) (Life Technologies). This serum-free media has L-glutamine added.
2. Sf-9 cells (Life Technologies). These cells come supplied in a cryogenic vial seeded at $1.5 \times 10^7$ cells/mL in 1.5 mL.
3. Fetal bovine serum (FBS) (Life Technologies).

## 3 Methods

### 3.1 Designing dsRNA

1. Determine which genes are of interest for silencing. The aforementioned websites will provide the information for both potential targets and nonspecific effects.
2. Design should make use of the websites listed under Subheading 2.1.
3. Once a target region is determined, a DNA template of that region has to be transcribed from the mRNA as described below.

4. In deciding on how much dsRNA you will need, a rule of thumb is 30 μg per well in a 6-well plate. For triplicate experiments that translates to 90 μg per treatment. Some genes can be silenced with far less dsRNA. The amount you will need will likely have to be determined experimentally, unless an amount can be found in the literature.

### 3.2 Making the Template DNA

1. Extract RNA from a culture of Sf-21 cells following the manufacturer's instructions given in the RNAqueous®-4PCR kit instruction manual (**steps 1–8**). For high yield and purity, it is recommended to use at least 500 μL of cells growing at $5 \times 10^5$ cells/mL. In manufacturer's **step 2**, lysing the cells by vortexing for 1 min is sufficient. In manufacturer's **steps 7** and **8**, use 50 μL of elution buffer (>90 °C) for the first elution and 15 μL of elution buffer (>90 °C) for the second elution.
2. Digest the RNA with DNAase 1 for 30 min at 37 °C (**step 1** of the optional DNAase digest).
3. Stop the DNAase 1 digest with DNAase inactivation reagent (manufacturer's **step 3** of optional DNAase digestion). Spin down the DNAase inactivation reagent at $10{,}000 \times g$ for 1 min (**step 4** of optional DNAase digestion). Transfer the RNA to a fresh 1.5 mL microfuge tube, being careful not to disrupt the DNAase inactivation reagent pellet on the bottom of the tube.
4. Aliquot 190 μL of nuclease-free water into a nuclease-free microcentrifuge tube. Measure the yield of RNA by carefully pipetting 10 μL of RNA into the 190 μL of nuclease-free water, mixing well, and measuring the absorbance at 260 nm and 280 nm (UV). The ratio of $Abs_{260}/Abs_{280}$ should be $\geq 2.0$. If this ratio is $< 2.0$, then the extraction was not successful and should be repeated. If the ratio is $\geq 2.0$, then calculate the yield of RNA by multiplying the $Abs_{260} \times 20$ (dilution) $\times 40$ to get the yield in ng/μL (RNA $Abs_{260}$ of 1 = 40 ng/μL).
5. Use 50–200 ng RNA per reaction with the Superscript™ III kit (Life Technologies) to make a cDNA template (reverse transcript) for PCR as per manufacturer's instructions (manufacturer's **steps 1–8** in the cDNA synthesis protocol that comes with the Superscript™ III kit) with the OligodT primers. Include RNAse H with the digestion (**step 7** of cDNA synthesis protocol). The entire protocol can be carried out in a programmable PCR thermocycler to insure consistency.
6. PCR amplify the cDNA to make the primary DNA template using Vent DNA polymerase (NEB) as per the manufacturer's instructions. The concentrations of components in the PCR vary depending on conditions. As a starting point, the following cocktail is recommended: 5 μL of reverse transcript from **step 5**, 2 μL 10× reaction buffer, 1 μL of each 10 μM primer

stock (not the T7 stock, that will be used later), 2 μL dNTP mix, 9 μL nuclease-free water, and 1 μL of Vent DNA polymerase. Typically, 28 cycles of PCR will yield sufficient template for subsequent PCR to add the T7 template.

7. Repeat the PCR amplification with the same conditions and the template made in **step 6** above to make the final DNA template with the T7 primer sequence. This time use the primers with the T7 sequence added. Use 5 μL of the PCR reaction from **step 6** above instead of the reverse transcript.
8. 32 cycles will yield an adequate amount of DNA template. The amount of dsRNA required should be calculated ahead of time as mentioned previously. For making 200–400 μg of dsRNA, two 20 μL PCR reactions will usually suffice.
9. Store amplified final DNA template briefly at 4 °C while thawing the kit components for the dsRNA synthesis. dsRNA should always be made with fresh PCR products.

### 3.3 In Vitro dsRNA Synthesis

1. Synthesize dsRNA by following manufacturer's instructions using the MEGAscript™ kit from Life Technologies (manufacturer's **steps 1–5**). Five reactions per dsRNA to be synthesized is recommended to obtain >500 μg dsRNA. Use 2 × 20 μL final DNA template (with T7 sequence added) and no water in assembling the reactions. This will give 40 μL template + 40 μL rNTP + 10 μL 10× buffer + 10 μL of enzyme mix = 100 μL reaction volume. The synthesis incubation should be extended to 5 or 6 h.
2. Extract the dsRNA using phenol–chloroform extraction following the MEGAscript™ kit instructions (manufacturer's "recovery of the RNA" **step 4**). The isopropanol precipitation should be carried out overnight at −20 °C. The pellet should be washed twice in −20 °C ethanol to increase purity following the overnight incubation in isopropanol. Use a pipet to remove as much ethanol as possible from the pellet. Vacuum drying of the pellet should be minimized to avoid drying the pellet. A dried pellet will be very difficult to resuspend.
3. Resuspend the pellet in 50 μL nuclease-free water and measure the $Abs_{260}$. Adjust the concentration to 3.3 μg/μL for best results when used for gene silencing.
4. Separate the dsRNA strands at 65 °C for 30 min by floating the microcentrifuge tube containing dsRNA in a water bath. Transfer some 65 °C water in a beaker to the benchtop and float the dsRNA tube in the water. Allow the beaker to come to room temperature. This will allow the dsRNA strands to slowly anneal.
5. Store the dsRNA at −20 °C.

### 3.4 Transfecting Sf-21 Cells in Culture

1. Seed Sf-21 cells in 2 mL Sf900 II SFM at $5 \times 10^5$ cells/mL in 6-well plates and allow to grow overnight at 27 °C in an incubator.
2. Make stock solutions of 15–30 μg dsRNA in 1 mL Sf900 II SFM for each replicate. The amount of dsRNA needed to silence a particular gene will have to be determined experimentally.
3. Make a stock solution of Sf900 II SFM + 10 % FBS. The volume of this stock solution should be 3 mL × number of wells in total experiment (treatments and controls).
4. Remove the 2 mL Sf900 II from each well of the 6-well plate from **step 1** and replace it with 1 mL of the dsRNA containing solution. Swirl wells to mix. Place the resulting 6-well plates in the incubator for 50 min.
5. Remove the 1 mL dsRNA-containing medium from the cells and replace with 3 mL of Sf900 II SFM + 10 % FBS (from **step 3** above).
6. Allow the cells to incubate for 36–72 h at 27 °C.
7. Assay for gene expression (*see* **Note 2**).

## 4 Notes

1. The in vivo dsRNA constructs were designed with an inverted repeat of the gene region targeted to synthesize dsRNA inside the cell. Silencing was comparable to that demonstrated using in vitro-synthesized dsRNA.
2. Assaying for gene expression should involve both an assessment of transcript and of protein expression. Simply looking at transcript levels does not give a clear indication of the state of the protein under study. Some proteins are more stable under physiological conditions than others; hence an assessment of protein presence or activity is needed.

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# Chapter 25

# Using the Baculovirus/Insect Cell System to Study Apoptosis

**Nor Chejanovsky**

## Abstract

Apoptosis is a physiological program of cell suicide conserved in invertebrates and vertebrates. Apoptosis is crucial to the normal development of organisms and in tissue homeostasis by promoting elimination of unwanted cells, including damaged or virus-infected cells. Due to the importance of programmed cell death for the survival of the organism, a tight regulation is exerted at various activation levels of the cell-death machinery. The utilization of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) to identify genes that inhibit the apoptotic process will be described using a transfection-based approach, illustrated by identification of the *p49* gene.

**Key words** Apoptosis, Baculovirus, Anti-apoptotic genes, Marker rescue, Polyhedra

## 1 Introduction

Apoptosis is a physiological program of cell suicide conserved in invertebrates and vertebrates that promotes the elimination of unwanted cells, including damaged- or virus-infected cells, and is fundamental for the normal development of organisms and in tissue homeostasis [1]. Due to the importance of programmed cell death for the survival of the organism, a tight regulation is exerted at various levels of activation of the cell-death machinery [2]. Animal viruses have evolved ways to evade, delay or suppress this important cell defense strategy [3–7]. Baculoviruses possess two types of genes, i.e., *iap* and *p35*-like genes, that can suppress apoptosis induced by virus infection or by diverse stimuli in vertebrates or invertebrates [8–11]. The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) expresses the *p35* gene during infection of *Spodoptera frugiperda* Sf-21 or Sf-9 cells, thereby suppressing their apoptotic response and allowing the completion of the viral replication cycle and polyhedra production [12, 13]. This fact, and the high conservation of the apoptotic

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machinery across the animal kingdom, can be implemented to isolate genes that suppress apoptosis from vertebrates and invertebrates [14–18]. This chapter describes a genetic-screening method to discover anti-apoptotic genes based on the transfection of an AcMNPV mutant null in *p35* and DNA libraries bearing putative anti-apoptotic genes. The methodology includes isolation of wild type and mutant AcMNPV DNA, induction and monitoring apoptosis in insect cells, a transfection-based marker rescue assay and utilization of the marker-rescue approach to evaluate structural motifs of apoptosis-suppressing proteins (aided by PCR-directed mutagenesis of the target protein). Insect cells constitute a useful model system to study apoptosis since they can easily be manipulated and grown. Moreover, the results and findings obtained using insect cells can be further tested in the whole organism [e.g., by infecting insects utilizing baculoviruses as vectors of expression or by using in vivo RNAi approaches (*see* Chapter 24)]. Finally, data obtained from the above experimental systems can be further elaborated and integrated with information available on insect genomes that have been completely sequenced (e.g., *Drosophila* and *Bombyx mori* [19, 20]) or are in the process of it (Lepidopteran genome project [21]).

## 2 Materials

### 2.1 Chemicals and Solutions

1. Restriction enzyme DpnI.
2. Ethanol absolute analytical grade diluted to 95 % (v/v) with sterile $ddH_2O$.
3. pIEx-1 plasmid (Novagen).
4. pBlueP49 stop plasmid [24].
5. Phenol–chloroform–IAA (isoamyl alcohol): a mixture of 25:24:1 parts of phenol, chloroform, and isoamyl alcohol, respectively.
6. Sodium acetate anhydrous diluted in sterile $ddH_2O$ to a 3 M solution.
7. Sodium chloride molecular biology grade.
8. Tfx™-20 (Promega).
9. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
10. TNM-FH serum-free medium.
11. Complete TNM-FH medium: TNM-FH serum-free medium, 10 % heat-inactivated fetal bovine serum, antibiotics (penicillin 50 units/mL and streptomycin 50 μg/mL).
12. Extraction buffer: 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.5 % SDS supplemented with 0.1 mg/mL proteinase K.

13. Oligonucleosome extraction buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 % sodium dodecyl sulfate (SDS) buffer containing 70 μg of proteinase K/mL.
14. PCR reaction mixture: 25 ng DNA, 2 μL 10× reaction buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 0.8 % (w/v) Nonidet-P40), 0.4 μL of each dNTP (10 mM), 1 μL of each primer (50 ng/μL), 0.2 μL Pfu-Taq Polymerase (1 U), $ddH_2O$ to a final volume of 20 μL.

### 2.2 Cells and Viruses

1. *S. frugiperda* Sf-9 and *Trichoplusia ni* Tn-368 cells maintained and propagated in complete TNM-FH medium.
2. Wild type (wt) *A. californica* multiple nucleopolyhedrovirus (AcMNPV) E-2 strain, *Spodoptera littoralis* nucleopolyhedrovirus SlNPV E-15 strain and vΔP35K/pol+, a mutant of AcMNPV lacking the *p35* gene [16].
3. vΔP35K/pol+ genomic DNA, i.e., DNA extracted from vΔP35K/pol+-infected cells.

## 3 Methods

### 3.1 Utilization of a Baculovirus-Based System to Discover Genes that Inhibit Apoptosis

Transfection of Sf-9 cells with genomic AcMNPV DNA enables the complete replication of this baculovirus, thereby resulting in formation of polyhedra in the cell nuclei that are easily detected using a light microscope (Fig. 1a). In contrast, transfecting cells with vΔP35K/pol+ genomic DNA results in extensive apoptosis (Fig. 1b). Thus, the ability of a gene to suppress apoptosis can be

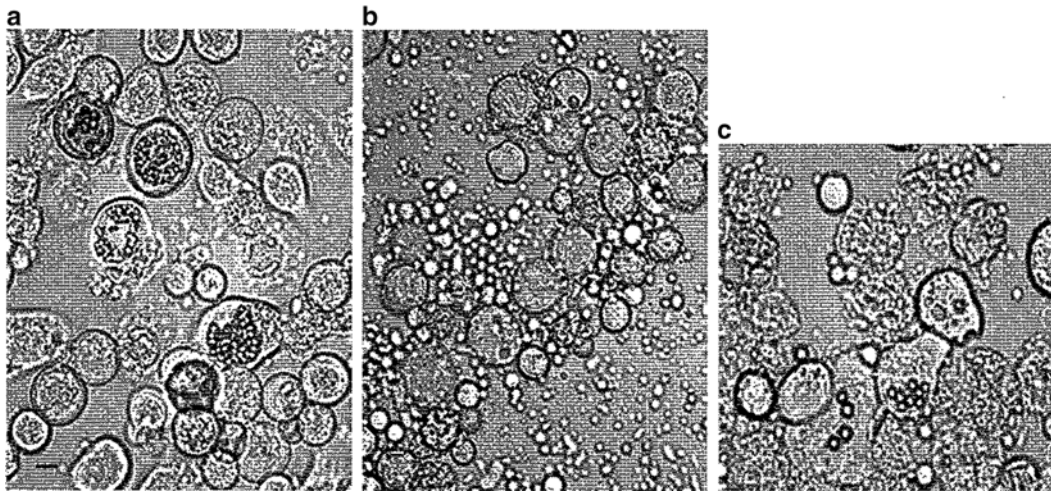


**Fig. 1** (**a** and **b**) Infection of Sf-9 cells with AcMNPV and vΔP35K/pol+ showing polyhedra-formation and apoptosis, respectively. (**c**) Sf-9 cells transfected with vΔP35K/pol+ and pAN (expressing *p49*) rescuing viral replication and showing polyhedra. Bar = 2 μm

assayed by co-transfecting Sf-9 cells with DNA (plasmid, cosmid, etc.) containing the gene of interest and vΔP35K/pol+ genomic DNA. Suppression of apoptosis rescues vΔP35K/pol+ replication and clear viral polyhedra can be seen in the nucleus of the transfected cells (Fig. 1c). This procedure was used to isolate *p49* (previously called *slp49*) from the SlNPV genome [16].

### 3.1.1 Infection of Insect Cells with Baculoviruses

1. Seed Sf-9 or Tn-368 cells in a 12 well plate ($5 \times 10^5$ cells/well, in 2 mL medium) and allow to attach at least for 4 h at 27 °C.
2. Replace the medium with the infecting viral inoculum (200 μL, of the desired viral MOI diluted in TNM-FH serum-free medium), added dropwise with gently rocking the plate (*see* **Note 1**).
3. After 1 h of adsorption, replace the medium with complete TNM-FH medium (same volume per well as in **step 1**).

### 3.1.2 Isolation of AcMNPV DNA

Wild type and mutant (*p35*-null) AcMNPV DNA are isolated from Sf-9 and Tn-368 cells, respectively, following infection with the corresponding viruses at an MOI of 0.5 (*see* **Note 2**).

1. Seed cells in a 60 mm dish ($2 \times 10^6$ cells in 4 mL complete TNM-FH medium) and allow to attach at least 4 h at 27 °C.
2. Prepare 1 mL virus inoculum containing $10^6$ pfu (0.5 pfu/cell $\times 2 \times 10^6$ cells) by diluting stock virus solution with TNM-FH medium (without serum) to infect the cells in the 60 mm dish at an MOI of 0.5.
3. Remove the medium from 60 mm dish (from **step 1**) and add the diluted virus inoculum (from **step 2**) dropwise while gently rotating the plate.
4. After 1 h of adsorption replace the medium with 4 mL of fresh TNM-FH complete medium and incubate the cells at 27 °C for 4–5 days.
5. Transfer the cell supernatants to 15 mL conical tubes and pellet the cells by centrifugation at $1000 \times g$ for 10 min at 4 °C.
6. Centrifuge the resulting cell supernatants (from **step 4**) at $12{,}000 \times g$ for 1 h at 4 °C to pellet the budded virus (*see* **Note 3**).
7. Resuspend the virus pellet in 350 μL extraction buffer and incubate at 37 °C for 4 h.
8. Extract the DNA samples with equal volumes of phenol–chloroform–IAA (isoamyl alcohol), mix by turning the tube up and down to avoid breaking the DNA.
9. Centrifuge at $12{,}000 \times g$ for 1 min and transfer the aqueous upper phase to a new sterile Eppendorf tube.
10. To the aqueous phase (from **step 9**) add 3 M sodium acetate (1/10 of the volume of the aqueous phase) and cold (−20 °C)

95 % ethanol (two volumes of the aqueous phase). Incubate at −20 °C for 15 min.

11. Centrifuge at 12,000 × *g* for 10 min.
12. Wash the pellet with cold (−20 °C) 70 % ethanol and air-dry briefly.
13. Resuspend in 100 μL TE buffer.
14. Quantify the amount of DNA by measuring the $OD_{260}$ of the solution (*see* **Note 4**).

### 3.1.3 Transfection Mixture Preparation

1. Solution A: To a polystyrene sterile tube add 200 μL TNM-FH serum-free medium. Add 3 μL Tfx-20.
2. Solution B: In a sterile Eppendorf tube add 200 μL TNM-FH serum-free medium. Add 1 μg of the relevant DNA (e.g., 500 ng of tested DNA and 500 ng vΔP35K/pol+ genomic DNA) (*see* **Note 5**).
3. Add Solution B to Solution A and mix gently (do not vortex).
4. Let the mixture stand for 30 min at room temperature before adding it to the cells.

### 3.1.4 Transfection

1. Seed Sf-9 cells ($5 \times 10^5$ cells/well) in a 12 well plate and allow to attach at least for 4 h at 27 °C (*see* **Note 6**).
2. Wash the cell monolayers two times with transfection medium (TNM-FH serum-free medium).
3. Add the transfection mixture containing the corresponding DNA dropwise while gently rocking the plate to avoid high local concentrations of the transfecting mixture that may result in cell lysis.
4. Incubate the transfected cells for 4 h at 27 °C and then replace the medium with 2 mL complete TNM-FH medium.
5. Incubate the cells at 27 °C for the period required by the experimental design (e.g., *see* Subheadings 3.1.5 and 3.1.9).

### 3.1.5 Induction of Apoptosis in Insect Cells by Baculovirus Infection

*S. frugiperda* cells can be induced to apoptosis by various stimuli (e.g., baculovirus infection, UV irradiation). We have utilized two approaches to induce apoptosis of Sf-9 cells: (1) infection with AcMNPV null mutant of *p35* (e.g., vΔP35K/pol+) (Subheading 3.1.5) and (2) transfection with the genomic DNA of a *p35* mutant (*see* Subheading 3.1.6).

1. Infect Sf-9 cells with vΔP35K/pol+ budded virus isolated in **step 5** of Subheading 3.1.2 (multiplicity of infection varying from 1 to 10; *see* Chapter 1).
2. Monitor the samples with light microscopy. Cell blebbing can be observed starting at about 12 h post-infection (pi) and increases with time (Fig. 1).

*3.1.6 Induction of Apoptosis in Insect Cells by Transfection*

1. Seed Sf-9 cells ($4 \times 10^5$/well as in Subheading 3.1.3, **step 1**) in a 12 well plate and allow to grow overnight at 27 °C.
2. Remove the medium and add the transfecting mixture prepared in Subheading 3.1.3 containing only vΔP35K/pol+ genomic DNA transfecting mixture. Rotate the plate while adding the transfecting mixture to avoid excessive local concentrations of the transfecting mixture (*see* **Note 6**). Replace the medium with complete TNM-FH at 2–4 h after transfection and incubate the cells for another 18–24 h at 27 °C.

*3.1.7 Monitoring Apoptosis by Direct Microscopic Observation of the Blebbing Cells*

Cell blebbing due to apoptosis is monitored by microscopic observation after overnight incubation. Eventually, about 60–80 % of the cells become apoptotic at 48 h (Fig. 1) (*see* **Note 7**).

*3.1.8 Monitoring Apoptosis by Extraction of Fragmented DNA (Oligonucleosomes)*

1. Extract DNA oligonucleosomes from virus-infected Sf-9 cells (e.g., infected with *p35*-null AcMNPV) with 400 μL oligonucleosome extraction buffer (Subheading 2.1) for 2 h at 37 °C as indicated in Subheading 3.1.2. Cells at 12–16 h post-infection (MOI = 5) are typically used.
2. Add 5 M NaCl (to a final concentration of 1 M) (i.e., 100 μL added to the 400 μL from **step 1**) and incubate the extracts overnight at 4 °C.
3. Treat the extracts with 500 μL phenol–chloroform–IAA (Subheading 2.1) (i.e., add to the 500 μL from **step 2**).
4. Separate the aqueous phase by centrifugation (12,000 × *g* for 2 min at room temperature) and transfer to a new Eppendorf tube.
5. Add 1 mL cold ethanol (−20 °C) to the aqueous phase obtained in **step 4** to precipitate the DNA and incubate at −80 °C for 15 min.
6. Centrifuge the sample at 12,000 × *g* for 15 min.
7. Rinse the pellet with 70 % cold ethanol (−20 °C) and centrifuge again.
8. Resuspend the DNA in 60 μL TE and analyze it by standard agarose electrophoresis using a 1 % gel (Fig. 2).

*3.1.9 Genetic Screen and Rescue of AcMNPV Replication*

1. 1 μg of vΔP35K/pol+ genomic DNA and 1 μg of test DNA (e.g., SlNPV cosmids) are co-transfected into $4 \times 10^5$ Sf-9 cells as described under Subheading 3.1.4 (*see* **Note 8**).
2. Examine the cells 3–4 days after transfection by light microscopy for the presence of polyhedra. In our example the transfection performed with cosmid C50 was positive in the assay and polyhedra could be seen in the cell nuclei (Fig. 3).

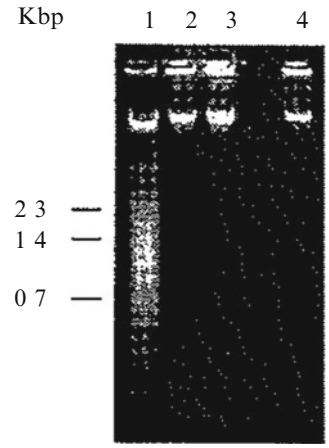


**Fig. 2** Oligonucleosomes extracted from Sf-9 cells infected with vΔP35K/pol+ (lane 1). Extracts from Sf-9 cells infected with AcMNPV (lane 2) and a recombinant vΔP35K/pol+ that bears the *p49* gene (lane 3). Mock infected cells (lane 4). Molecular markers are indicated on the *left*. Reproduced from ref. 16 with permission of ASM journals

*3.1.10 Identification of the Apoptosis-Suppressing Gene*

This is achieved by subcloning the tested DNA (if necessary) (Fig. 3).

1. Subclone by standard methods the high molecular weight DNA (e.g., cosmid C50) into various plasmids and repeat the transfection procedure from above.
2. The polyhedra positive plasmids are further subcloned and finally the sequence of the smallest rescuing plasmid is determined (Figs. 1 and 3).
3. Further confirmation that the identified gene is functional can be obtained by introducing mutations and assaying the mutant plasmids in the marker-rescue assay (*see* Subheading 3.3).

*3.1.11 Isolation of Recombinant Baculoviruses*

Recombinant baculoviruses bearing the new apoptosis suppressor gene can be isolated since recombination occurs during baculovirus replication.

1. Perform the rescue assay by transfecting Sf-9 cells with a plasmid DNA, bearing the apoptosis suppressor gene (e.g., pAP, Fig. 3), and vΔP35K/pol+ genomic DNA as described under Subheading 3.1.9.
2. Four days later take the supernatant of the cells containing budded viruses, perform plaque assay and isolate polyhedra-positive plaques (*see* Chapter 4).
3. Take the plaque with a sterile Pasteur pipette or a micropipette and place it in an Eppendorf tube containing 1 mL complete TNM-FH medium and vortex.

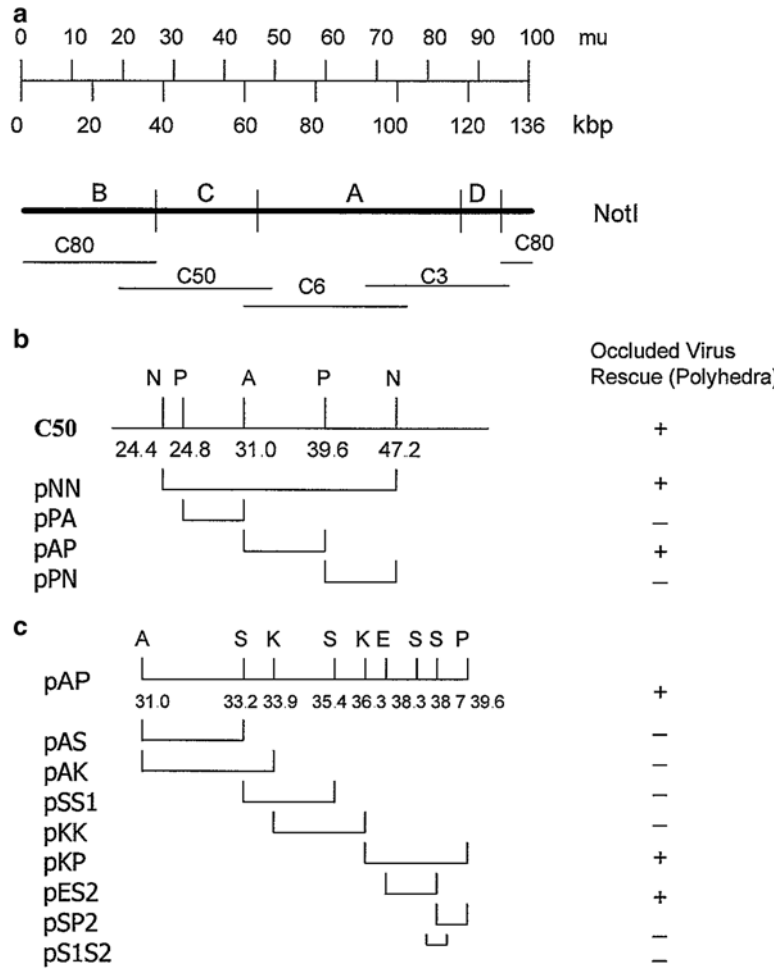


**Fig. 3** Rescue of AcMNPV occluded viruses by cosmids and plasmids bearing SlNPV DNA fragments. (**a**) Not I linear restriction map of SlNPV. The scales indicate SlNPV map units (m.u.) and kilobase pairs (kbp). The bars below (C80, C50, C6, and C3) represent the various overlapping cosmids of the genomic SlNPV cosmid library. (**b**) Restriction map of the cosmid C50 and individual plasmid subclones indicating their ability to rescue or not (+ and –, respectively) the replication of vΔ35K/pol+, as detected by the presence of polyhedra in the nuclei of Sf-9 cells co-transfected with vΔ35K/pol+ and plasmid DNA. *N* NotI, *P* PstI, *A* ApaI. (**c**) Restriction map of the ApaI-PstI region corresponding to SlNPV 31.0–39.6 m.u. *S* SalI, *K* KpnI, *E* EcoRI. Also, subclones able to rescue vΔ35K/pol+ polyhedra formation as in (**b**) are indicated. Reproduced from ref. 16 with permission of ASM journals

4. Repeat the plaque assay using the supernatant from **step 3**.
5. Repeat the plaque purification once more and amplify the pure plaque by infecting Sf-9 cells.
6. Confirm that your recombinant baculovirus bears the anti-apoptotic gene by PCR using gene-specific primers.

### 3.2 Utilization of Baculovirus-Based Plasmids to Extend the Applicability of the Assay to Discover Anti-apoptosis Genes in Vertebrates and Invertebrates

The same approach from Subheading 3.1 can be utilized to identify functional apoptosis suppressor genes from other invertebrate and vertebrates. For this purpose two requirements need to be fulfilled:

1. The investigated gene can be expressed in the insect cells.
2. The investigated gene is functional in the insect cells.

The first condition can be achieved by placing the desired gene under the control of a promoter that is active in the insect cells, e.g., the *ie1* promoter available in the pIEx-1 plasmid or the Hsp70 promoter of *Drosophila* [17] and performing the marker-rescue assay described under Subheading 3.1.9.

### 3.3 Evaluating Structural Motifs of Apoptosis-Suppressing Proteins

The transfection rescue approach described under Subheading 3.1 can be used to learn about the relationship between the structural motifs of the expressed protein and its function. For this purpose we assayed for the ability of site-directed mutants in various structural motifs of P49 to rescue apoptosis in the above marker rescue assay. We modeled the three-dimensional structure of P49 utilizing Swiss-Model [22] and 3D-PSSM Web server Biomolecular Modeling Laboratory at The Imperial Cancer Research Fund [23] and introduced site directed mutations in P49 using the overlap extension polymerase chain reaction with complementary primers containing the desired mutation in the plasmid pBlue-49-stop [24] (*see* Fig. 4 and Table 1). The method is described below.

#### 3.3.1 Site-Directed Mutagenesis Using Overlap Extension Polymerase Chain Reaction

1. Use as template plasmid DNA bearing the identified gene (e.g., pBlue-P49 stop).
2. Select primers of about 25–30 mers: one pair of complementary primers of equal length for each mutant with six overhanging non complementary bases at the 3′ terminus of each primer to enable better stability at the transformation step (*see* **Note 8**).
3. Adjust the reaction conditions to 1 cycle of 5 min at 95 °C, 18 cycles of 30 s at 92 °C, 30 s at 58 °C, and 60–90 s at 72 °C (*see* **Note 9**).
4. Following PCR, the reaction products are digested with DpnI (0.5 U) at 37 °C for 1 h. DpnI cuts the parental methylated DNA, leaving intact the amplified plasmid.
5. Transform *E. coli* XL1 Blue with the resultant amplified plasmid using standard techniques.
6. Select and amplify three to four colonies.
7. Determine the sequence of the mutated plasmid DNA. Usually, more than 80 % of the colonies bear the introduced mutation.

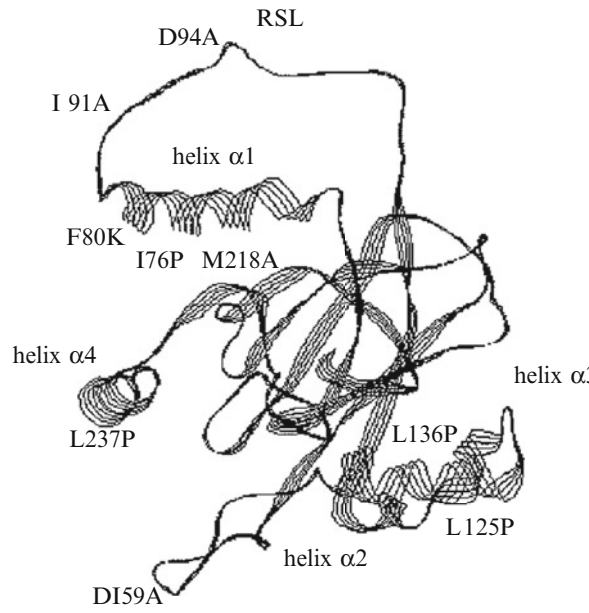


**Fig. 4** Partial three dimensional structure of P49 (amino acid residues 1–299), showing predicted several domains important for its function: (a) β-core, composed of a β-barrel domain with a large insertion that forms the reactive site loop (RSL). The RSL begins at the amphipathic α1 helix (between Val 69 and Phe 83) and traverses the β-sheet central region exposing the D 94 residue at the apex, in the context of the putative caspase-cleavable motif 91 TVTD G 95 and follows downwards rejoining the β-barrel; (b) three additional alpha helical domains, α2 (between Gly 115 and Asn 127), α3 (between Tyr 133 and Pro 146), and α4 (between Ile 231 and Arg 236); (c) a side loop between amino acids Leu 147 and Lys 167. Several mutations introduced are indicated. The effect of the above mutations on P49 function is shown in Table 1. Adapted from ref. 24

*3.3.2 Quantitative Evaluation of the Marker Rescue Assay*

1. Transfect each plasmid containing the desired mutation with vΔP35K/pol+ genomic DNA into Sf-9 cells as described under Subheading 3.1.9.
2. Evaluate microscopically the ability of the plasmids to allow polyhedra formation.
3. Collect supernatants of the transfections and perform plaque assays as described in Chapter 4. Mutants that yield more than 60,000 non-apoptotic polyhedra-positive plaque forming units are considered functional, whereas mutations that yield less than 8000 plaques are considered nonfunctional (*see* **Notes 10** and **11**).

**Table 1**
**Structural domains important for P49 function**

| Predicted domain | Mutation | Functional effect[a] (Apoptosis suppression) |
|---|---|---|
| helix α1 | L76P | – |
| helix α1 | F80K | – |
| helix α2 | L125P | – |
| helix α3 | L136P | + |
| helix α4 | L237P | – |
| β-sheet | M218A | – |
| Turn | D159A | + |
| Reactive site loop (RSL) | T91A | – |
| Reactive site loop (RSL) | D94A | – |

[a]The ability of the various P49 mutants to rescue the replication of vΔP35K/pol+ was determined by co-transfecting them with genomic vΔP35K/pol+ DNA to Sf-9 cells. Rescuing mutants (+) yielded more than 60,000 non-apoptotic polyhedra-positive plaque-forming units/mL, whereas non-rescuing mutants (–) yielded less than 8000 plaque-forming units/mL. The helical motifs α1, α2 and α4, the residue M218 and the residues T91 and D94 of the RSL are necessary for the antiapoptotic function of P49 (adapted from ref. 24)

## 4 Notes

1. The original virus stock is kept at high titer in medium containing serum. By diluting in serum-free medium the adsorption of the virus particles to the cells is improved.
2. Tn-368 cells do not undergo apoptosis by infection with vΔP35K/pol+ budded virus and are suitable to isolate genomic DNA of this virus. Also, addition of the caspase inhibitor 100 mM N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk; MP Biomedicals) may improve the infection [13].
3. The clarified supernatant can be kept at 4 °C as a source of budded virus stock.
4. The ratio of $OD_{260}/OD_{280}$ should be closer to 1.8. Much lower values indicate that the extracted DNA is contaminated by organic material or protein. This requires repeating the extraction procedure.

5. Maintain a ratio of 3 μL Tfx-20 per 1 μg of total DNA to be transfected.
6. Transfection efficiency will be higher if the cell density is not confluent, about 60–80 % confluency is recommended.
7. It is very important that the transfection mixture be prepared gently following the given directions. Otherwise, some cell lysis and blebbing can occasionally be observed due to excessive local concentrations of the transfecting reagent. A good practice is to transfect cells with wild type AcMNPV genomic DNA as a control that expresses *p35* and should not show apoptosis.
8. For example, to prepare the mutant D94A in *p49* we utilized the pair of primers, 5′-CGACCGTGACCGCTGGCGGTGG AGCCGAT-3′ and 5′-CTCCACCGCCAGCGGTCACGGTC GCCGAT-3′ (the base change is underlined).
9. The conditions of the annealing temperature are adapted to each pair of primers.
10. Perform this transfection in triplicate to increase accuracy.
11. This is required since some mutants may show low performance due to partial loss of function.

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# Chapter 26

# Generation of Envelope-Modified Baculoviruses for Gene Delivery into Mammalian Cells

**Christian Hofmann**

## Abstract

Genetically modified baculoviruses can efficiently deliver and express genes in mammalian cells. The major prerequisite for the expression of a gene transferred by baculovirus is its control by a promoter that is active in mammalian cells. This chapter describes methods for producing second generation baculovirus vectors through modification of their envelope. Envelope modified baculoviruses offer additional new applications of the system, such as their use in in vivo gene delivery, targeting, and vaccination. Methods of generating a recombinant baculovirus vector with a modified envelope and its amplification and purification, including technical scale production, are discussed. A variety of notes give clues regarding specific technical procedures. Finally, methods to analyze the virus and transduction procedures are presented.

**Key words** Baculovirus, Gene delivery, Targeted vectors, Complement-resistant, Vaccine, Mammalian cell, Envelope modification, Hepatocyte, Vector generation, Gene delivery

## 1 Introduction

Twenty years ago, we genetically engineered baculoviruses that can efficiently deliver and express genes in mammalian cells [1]. The only prerequisite for the expression of a gene transferred by baculovirus is its control by a promoter that is active in mammalian cells. Such recombinant baculovirus vectors have been used successfully for a variety of applications, including gene transfer studies, generation of monoclonal antibodies, and cell-based assays [2]. The advantages of recombinant baculovirus vectors for mammalian cell expression are their safety due to their "non-mammalian-virus-based" origin. Baculoviruses have the unique property of replicating in insect cells while being incapable of initiating a replication cycle and infectious virus in mammalian cells. The viruses can be readily manipulated and easily generated to high vector titers. They show no cytopathic effect in mammalian cells and have a large capacity for the insertion of foreign DNA. The recent generation of envelope modified baculoviruses offers

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additional potential applications of the system, including in vivo gene delivery, targeting, and vaccination. The methods for the development of recombinant envelope-modified baculoviruses are described.

### 1.1 Principle of Baculovirus Envelope Modification

The main baculovirus envelope protein, gp64, is responsible for virus uptake by insect cells and hepatocytes [3]. Baculoviruses deleted of their natural single copy gp64 gene cannot be productively propagated in insect cells. Therefore, a strategy to modify the baculovirus envelope is to insert the modified gp64 gene as an additional copy into the baculovirus genome. A site for modification of this additional gp64 gene that successfully leads to display of the modification on the virus surface is the N-terminus in between the gp64 signal sequence and mature gp64 [4]. The modified gp64 can be adequately co-expressed in insect cells, even from the late polyhedrin promoter, during virus amplification and assembles into the viral envelope.

### 1.2 Selection of the Envelope Sequence Based on Intended Use

#### 1.2.1 Complement-Resistant Vectors

Gene delivery with baculovirus vectors in vivo is hampered by the complement system [5–7]. However, a variety of proteins are described that may protect the virus from complement-mediated inactivation. We selected a functionally active sequence of human decay acceleration factor (DAF) without signal sequence and with the glycosyl-phosphatidylinositol (GPI)-anchor (to insert the resulting protein into the viral membrane) to protect the baculovirus from complement. This sequence was cloned in between an extra copy of the gp64 signal sequence and mature gp64 and inserted into the polyhedrin locus to generate complement-resistant vectors [8, 9].

#### 1.2.2 Targeted Vectors

As described under Subheading 1.1, sequences for specific receptor ligands can also be inserted to generate targeted vectors and to expand the host range of baculovirus vectors. We have generated envelope-modified baculovirus vectors displaying the epidermal growth factor on the virus surface and achieved an enhanced gene delivery into EGF-receptor positive cells in comparison with vectors having the wild-type envelope. In vivo tumor targeting of a systemically administered, tropism-modified baculoviral vector was also shown recently and highlights the potential of baculovirus-mediated targeted therapies [10].

#### 1.2.3 Vaccine Vectors

The unique possibility of baculovirus vectors to modify the envelope and simultaneously express genes from the viral backbone in mammalian cells may end up in new vaccines [11–14]. Antigenic sequences can be displayed on the viral surface to trigger a humoral immune response and other immunogenic sequences can be expressed to induce a cellular immune response.

## 2 Materials

### 2.1 Transfer Plasmid for Envelope Modification and Mammalian Gene Expression

1. Baculovirus transfer plasmid pBACsurf-1 (EMD Biosciences, San Diego, CA).
2. Reagents for PCR, including specific primers with restriction site overlaps for *Sma*I, *Kpn*I, or *Pst*I.
3. Enzymes for manipulation of DNA (restriction endonucleases: *Sma*I, *Kpn*I, or *Pst*I and *Eco*RV, Klenow enzyme, T4 DNA ligase, etc.).
4. Luria–Bertani (LB) medium.
5. Agar plates.
6. Selection agent (ampicillin).
7. Competent *E. coli* cells DH5-alpha (Invitrogen, Carlsbad, CA).
8. Kits and/or solutions for preparation of plasmid DNA from *E. coli* (Qiagen, Inc., Valencia, CA).
9. Kits and/or solutions for gel purification of DNA fragments (Qiagen, Inc., Valencia, CA).
10. Phenol–chloroform–isoamylalcohol solution (25:24:1).

### 2.2 Insect Cell Culture

1. Sf-9 cells (PharMingen, EMD Bioscience, San Diego, CA).
2. EX-CELL™ 401 medium (JHR Bioscience, Lenexa, KS).
3. Complete EX-CELL™ 401 medium: EX-CELL™ 401 medium, 10 % heat-inactivated fetal calf serum and 100 mg/L streptomycin and 100,000 units/L penicillin.
4. Tissue culture plates and flasks.
5. Cellspin-platform and spinner flasks (Integra Bioscience, Fernwald, Germany; distributed in the USA by Argos Technologies Inc., East Dundee, IL).

### 2.3 Virus Generation

1. Insect cell culture material, *see* Subheading 2.2.
2. Baculovirus genomic DNA, BaculoGold™ (PharMingen, EMD Biosciences, San Diego, CA).
3. Recombinant transfer plasmid from Subheading 3.1 (pBacsurf_X_mam).
4. Falcon polystyrene tubes.
5. Lipofectamine (Gibco, Carlsbad, CA).
6. Sterile distilled water.

### 2.4 Titration by Plaque Assay

1. Insect cell culture material, *see* Subheading 2.2.
2. SeaPlaque-Agarose™ (FMC, Rockland, USA).
3. Sterile distilled water.
4. 60 mm plates.
5. Plastic box.

### 2.5 Amplification of Recombinant Viruses

1. Insect cell culture material, *see* Subheading 2.2.
2. Recombinant virus stock or single plaque virus (**step 15** under Subheading 3.3.2).
3. 1 L spinner flasks (Cellspin, Integra, Fernwald, Germany; distributed in the U.S. by Argos Technologies Inc., East Dundee, IL).

### 2.6 Virus Concentration and Purification

1. Virus stock to be concentrated and purified.
2. Large capacity ultracentrifuge tubes, e.g., 38 mL polyallomer ultracentrifuge tubes for SW28 rotor, Beckman.
3. Ultracentrifuge tubes with a small diameter, e.g., 17 mL polyallomer ultracentrifuge tubes for SW40 rotor, Beckman.
4. Phosphate-buffered saline (PBS): 1 mM $Na_2HPO_4$, 10.5 mM $KH_2PO_4$, 140 mM NaCl, 40 mM KCl, adjust pH to 7.4 through addition of 1 M HCl.
5. Sucrose cushion solution: 25 % sucrose (w/w) in PBS, pH 7.4.
6. Sucrose gradient solutions: 25 % sucrose (w/w) in PBS and 60 % sucrose (w/w) in PBS, pH 7.4.
7. Sucrose-gradient-former (Jule Inc., Milford, CT).
8. Rotors and ultracentrifuge for centrifugation of up to 96,000 × *g*, e.g., SW28 and SW40 rotor, Beckman.

### 2.7 Virus Analysis

#### 2.7.1 Immunoblotting

1. A4p virus preparation (Subheading 3.3.5).
2. Sample buffer: 62.5 mM Tris–HCl, pH 6.8, 10 % glycerin, 2 % SDS, 300 mM 2-mercaptoethanol, 0.0025 % bromophenol blue.
3. SDS polyacrylamide gels (8 %).
4. 1 mM Dithiothreitol (DTT).
5. Rainbow molecular weight protein marker (Amersham (now part of GE Healthcare), Piscataway, NJ).
6. Western-blot apparatus (Owl Scientific, Portsmouth, NH).
7. Semidry-blotter (Serva Electrophoresis GmbH, Heidelberg, Germany; Crescent Chemicals Corp., Hauppauge, NY).
8. Transfer buffer: 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 5 % (v/v) methanol.
9. Hybond-C extra membranes (Amersham Pharmacia Biotech, Freiburg, Germany; Amersham (now part of GE Healthcare), Piscataway, NJ).
10. Blocking buffer: 5 % (w/v) milk powder, 0.1 % (v/v) Tween 20 in PBS.
11. Primary antibody against AcV5 [15] or directed against your specific protein displayed on the surface.

12. Horseradish peroxidase conjugated antisera (Pierce, St. Augustin, Germany; Rockford, IL).
13. ECL detection system (Amersham Pharmacia Biotech, Freiburg; now a part of GE Healthcare, Piscataway, NJ).

*2.7.2 Electron Microscopy*

1. A4p virus preparation.
2. Carbon coated grids (Agar Scientific Limited, Stansted Essex; Ted Pella, Inc., Redding, CA).
3. Monoclonal primary antibody.
4. 10 % (w/v) bovine serum albumin (BSA).
5. PBS.
6. 12 nm colloidal Gold-AffiniPure goat anti-mouse IgG (Dianova, Hamburg, Germany).
7. Phosphotungstic acid (PTA).
8. Transmission electron microscope (e.g., 902A, Zeiss, Oberkochen, Germany).

## 3 Methods

### *3.1 Transfer Plasmid for Envelope Modification and Mammalian Gene Expression*

*3.1.1 Basic Vector Elements*

The baculovirus transfer plasmid pBACsurf-1 is designed for in-frame insertion of DNA-sequences between the gp64 signal sequence and the mature protein coding sequence (under the control of the polyhedrin promoter). Expressed fusion proteins are incorporated onto the virion surface, anchored by the transmembrane domain of gp64 [4]. Mammalian expression cassettes can be inserted into the *Eco*RV-site (Fig. 1).

*3.1.2 Insertion of a Mammalian Expression Cassette*

Use standard cloning procedures to generate pBacsurf_mam by inserting an expression cassette that is active in mammalian cells blunt-end into the *Eco*RV-site of pBacsurf-1 (*see* **Note 1**).

*3.1.3 PCR-Cloning of New Envelope Sequence*

PCR-amplify the sequence to modify the baculovirus envelope and insert it via *Sma*I or *Kpn*I or *Pst*I into pBACsurf_mam to generate pBACsurf_X_mam (*see* **Note 2**).

*3.1.4 Baculovirus Genomic DNA*

BaculoGold™ is a modified *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) Baculovirus DNA that contains a lethal deletion and does not code for viable virus. Co-transfection of the BaculoGold-DNA with a complementing Baculovirus Transfer plasmid, e.g., pBACsurf_X_mam (Subheading 3.1.3), rescues the lethal deletion after homologous recombination in insect cells (Fig. 1).

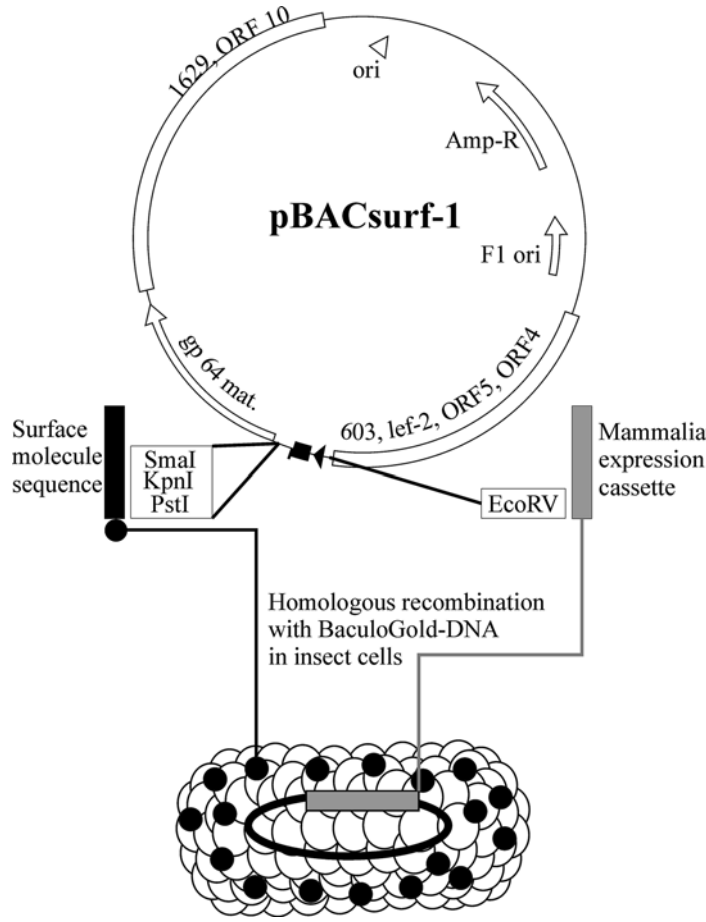


**Fig. 1** Map of baculovirus transfer plasmid pBACsurf-1 with insertion sites for envelope modification and expression of genes in mammalian cells. Viruses are generated by homologous recombination with the transfer vector and genomic baculovirus DNA in insect cells. *Black arrow*: polyhedrin promoter; *Black box*: gp 64 signal sequence; gp 64 mat.: gp 64 mature domain; *Open boxes*: 1629, ORF 10 and 603, lef-2, ORF5, ORF4: baculovirus genomic sequences/genes. *Sma*I, *Kpn*I, and *Pst*I are restriction sites to insert surface modification sequences; *Eco*RV can be used to insert a mammalian expression cassette

### *3.2 Insect Cell Culture, Serum-Free Versus Serum Containing*

In general, baculovirus can be produced in serum-free EX-CELL™ 401 medium or complete EX-CELL™ 401 medium. We recommend using complete EX-CELL™ 401 medium at least until large-scale production in spinner flasks (through **step 17** under Subheading 3.3.3). The use of serum-free medium facilitates purification because of the absence of FCS, but results, at least in our hands, in about 1 log less virus output compared to serum-containing medium.

### 3.3 Virus Generation

#### 3.3.1 Producing the Recombinant Virus by Homologous Recombination in Insect Cells

1. Prepare and label two 25 $cm^2$ tissue culture flasks with $2 \times 10^6$ Sf-9 cells (50–70 % confluent). One flask will be the experimental co-transfection flask, the other a negative control flask.
2. After cell attachment, wash cells gently 3 times with 3 mL EX-CELL™ 401 medium. Finally, add 1 mL EX-CELL™ 401 medium.
3. Experimental co-transfection: Combine 0.5 μg BaculoGold™ DNA and 3 μg recombinant Baculovirus Transfer Vector in a *Polystyrol* tube, add sterile water to a total of 50 μL and mix by pipetting up and down (*see* **Note 3**).
4. Mix 20 μL Lipofectamine and 30 μL sterile water in a separate Polystyrol tube.
5. Add DNA solution (from **step 3**) drop by drop to Lipofectamine–water mixture (from **step 4**).
6. Incubate mixture for 15 min at room temperature (becomes slightly opalescent).
7. Add the mixture from **step 6** drop by drop to the experimental co-transfection flask (from **step 2**). Gently rock the flask back and forth to mix the drops with the medium.
8. Incubate the two flasks at 27 °C for 4 h.
9. After 4 h, remove the medium from the experimental and the negative control flasks. Add 5 mL complete EX-CELL™ 401 medium and incubate the plates at 27 °C for 5 days.
10. After 5 days, cells of the experimental co-transfection flask will have stopped dividing and will often float in the medium. Cells in the control flask will be 100 % confluent.
11. Collect now the virus-containing supernatant of the experimental co-transfection flask and store at 4 °C in the dark.
12. Baculovirus-containing supernatant will be used for isolation of single recombinant viruses by plaque assay.

#### 3.3.2 Isolation of Single Recombinant Viruses, Titration, and Storage

1. Seed Sf-9 cells on 60 mm plates ($2 \times 10^6$ cells per plate using a sufficient volume of medium to totally cover the surface). Seed up to 7 plates (for the virus dilutions $10^{-2}$ to $10^{-8}$—*see* **step 2**). Allow the cells to attach for at least 10 min. It is important that this is done on a level surface to allow the cells to spread evenly over the bottom of the plate.
2. Prepare serial dilutions ($10^{-2}$ to $10^{-8}$) of virus-containing supernatant (e.g., from **step 12** under Subheading 3.3.1) in 1 mL complete EX-CELL™ 401 medium.
3. Aspirate medium from **step 1** and immediately add respective dilution from **step 2**.

4. Incubate the plates at 27 °C for 1 h to allow virus particles to infect the cells.
5. While the cells are incubating, prepare a 0.8 % SeaPlaque-Agarose in complete EX-CELL™ 401 medium. Make up 4 mL for each 60 mm tissue-culture plate to be overlaid: Prepare a solution of 4 % SeaPlaque-agarose in sterile distilled water and autoclave. Alternatively, melt in advance prepared by microwaving. Heat complete EX-CELL™ 401-medium to 60 °C in a water bath. Cool the melted SeaPlaque-Agarose to 60 °C in a water bath. Dilute the SeaPlaque-agarose to 0.8 % (w/v) with heated complete EX-CELL™ 401-medium and cool the 0.8 % agarose–medium mixture to 40 °C.
6. Remove the virus inoculum from cells and take care that the monolyer does not dry out.
7. Overlay cells with 4 mL of the 0.8 % agarose/complete EX-CELL™ medium mixture.
8. Allow plates to sit undisturbed on a level surface until agarose hardens (about 20 min).
9. Plates should be kept inverted in a humid atmosphere (e.g., a plastic box with wet paper) at 27 °C.
10. Plaques develop after about 5–10 days and can be visualized and counted by inverting the plates on a dark background.
11. Determine the titer by recalculating the number of counted plaques with the respective dilution.
12. Isolate three single plaque recombinant viruses from a "high" dilution plate by picking with a pipette into a plaque and transferring virus in agarose into an Eppendorf tube prefilled with 1 mL complete EXCELL™-medium.
13. Allow virus to diffuse out of the agarose for 1 h at room temperature, mix and divide into two aliquots. Label the tubes as A0 (amplification zero, any additional amplification will produce higher stock numbers, e.g., A1, A2, A3).
14. Freeze one (back-up) aliquot (500 µL) at −80 °C (*see* **Note 4** and Chapter 9).
15. The other aliquot can be stored at 4 °C in the dark until amplification.

### *3.3.3 Amplification of Recombinant Viruses*

1. For each virus from **step 13** under Subheading 3.3.1 seed $2 \times 10^6$ Sf-9 cells (50–70 % confluent) into a 25 $cm^2$ tissue culture flask. Also seed a control flask that will not be infected with virus. Use a sufficient volume to totally cover the flask surface. Allow the cells to attach for 15 min, remove the medium, and add 4.5 mL fresh complete EX-CELL™ 401 medium.
2. Add the 500 µL of the low titer A0 recombinant stock (**step 13** under Subheading 3.3.1) to a flask.

3. Incubate the cells at 27 °C for 5 days. Check for signs of infection 3 days post-infection (pi) (floating cells in comparison to a confluent monolayer in the control flask).
4. Harvest the supernatant from the flask and centrifuge at 500 × *g* for 15 min to remove cellular debris.
5. Store the virus supernatant (A1) in a sterile tube at 4 °C in the dark and freeze 500 µL at −80 °C as A1-back-up (*see* **Note 4**).
6. At this stage, rather than determining the virus titer, which is normally around $10^6$–$10^7$ pfu/mL, try to amplify your specific inserts by PCR using 1 µL (heated for 1 min at 95 °C) of your virus-containing insect cell supernatant as template for each reaction.
7. For further amplification, seed $6 \times 10^6$ Sf-9 cells (50–70 % confluent) into 75 $cm^2$ tissue culture flasks. Allow them to attach for 15 min and change to 3 mL fresh complete EX-CELL™ 401 medium. Add 1 mL of your A1 stock (PCR-positive for the inserts) and incubate for 1 h at on a rocking plate.
8. Add 11 mL fresh complete EX-CELL™ 401 medium and incubate at 27 °C for 3–5 days until nearly all cells are floating (in comparison to the control flask).
9. Harvest the supernatant from the flask and centrifuge to remove cellular debris (500 × *g*, 15 min).
10. Store the virus supernatant (A2) in a sterile tube at 4 °C in the dark and freeze 500 µL at −80 °C as A2-back-up (*see* **Note 4**).
11. Proceed with amplification (**steps 7–10**) by using three 225 $cm^2$ tissue culture flasks (i.e., scale-up cells and virus by a factor 3) to end up with 135 mL virus stock A3.
12. Titrate the A3 stock by plaque assay (**steps 1–11** under Subheading 3.3.2). The titer should reach about $1–2 \times 10^8$/mL at this stage.
13. Amplification in spinner flasks: Seed approximately $5 \times 10^5$ Sf-9 cells/mL in a total of 250 mL medium in a 1 L spinner flask. The cells should be healthy (95–100 % viable) and have a population doubling time of ~24 h (*see* Chapter 1).
14. Cultivate cells (*see* Subheading 3.2) for about 2–3 days until a cell density of $2–3 \times 10^6$/mL is reached (exponential growth phase; *see* Chapter 1) and then add A3 virus stock at an MOI of 1. Repetitive infections with an MOI of substantially higher than 1.0 will select for deletion mutants that may no longer contain your inserts.
15. Cultivate cells for an additional 1.5–2.5 (absolute maximum) days. Check the progress of infection by examining aliquots of the culture under the microscope. Aliquots should also be used to monitor virus production (plaque assay) over time (*see* **Note 5**).

16. To harvest the virus, first pellet cells by centrifugation at 500 × *g* for 15 min and then centrifuge the resulting supernatant at 5000 × *g* for 15 min. Virus containing insect cell medium should be clear (*see* **Note 5**).
17. Titrate the A4 by plaque assay (**steps 1–11** under Subheading 3.3.2) and store at 4 °C in the dark until purification.

#### *3.3.4 Virus Concentration*

1. Load virus stock (A4) to high capacity polyallomer ultracentrifuge tubes.
2. Underlay with the sucrose cushion solution (10 % of the total volume of the virus stock).
3. Centrifuge at 80,000 × *g* for 75 min at 4 °C.
4. Decant the supernatant and carefully remove all liquid. The virus pellet should be translucent white.
5. Resuspend the pellets in a total of 2 mL PBS, retain an aliquot for titration to monitor a potential virus loss and purify as described under Subheading 3.3.5.

#### *3.3.5 Virus Purification (See **Note 6**)*

1. For 2 mL concentrated virus (**step 5** under Subheading 3.3.4), pour two 14 mL 25–60 % linear sucrose gradient into a 17 mL polyallomer ultracentrifuge tube.
2. Carefully load the concentrated virus (1 mL/tube) (**step 5** under Subheading 3.3.4) onto the gradient.
3. Centrifuge at 96,000 × *g* for 3 h at 4 °C.
4. Collect the white band (budded virus) in a minimum volume (at around 47–49 % sucrose).
5. Dilute the virus-bands about tenfold.
6. Centrifuge at 80,000 × *g* for 75 min at 4 °C.
7. Decant the supernatant and remove any liquid.
8. Resuspend the pellet in a small volume (1–2 mL) of PBS, aliquot (A4p, "A4purified") and freeze at –80 °C before titration.

### *3.4 Virus Analysis*

Virus analysis is an important quality aspect to compare, e.g., newly acquired features of envelope-modified viruses to vectors with wild-type envelope. Besides the three analytical methods described in this section, a Southern-blot analysis should be performed with every large scale preparation of purified baculoviruses to confirm insert integrity. In addition, a Southern-blot analysis allows for calculating the total number of baculovirus genomes in a given sample that includes both infectious and noninfectious viruses. Dividing this number by the number of infectious viruses determined from plaque assay experiments yields the particle to infectious virus ratio. The smaller this ratio, the better is your preparation.

### 3.4.1 Functional Assays

Functional assays to determine envelope-modified virus integrity depend on the specific construct (*see* Subheading 1.2) and should be performed with A4p preparations (**step 8** under Subheading 3.3.5). For example, we generated complement-resistant vectors by incorporating decay acceleration factor (DAF) into the viral envelope. In addition, the vectors were able to mediate ß-galactosidase expression in insect and mammalian cells. The functionality was therefore tested in an Sf-9 insect cell-based and an hepatocyte-based assay [8] by incubating the viruses with active complement. Targeted vectors can be assessed by their eventually acquired new function to bind to cells or to mediate gene delivery in previously non-susceptible cell lines or respective animal models [10]. Vaccine vectors can be tested based on their ability to mediate a humoral and/or cellular immunity against displayed or expressed antigens after injection into animals [11–14].

### 3.4.2 Immunoblotting

In order to determine the composition of envelope modification, vectors from A4p preparations (**step 8** under Subheading 3.3.5) with wild-type envelope and modified envelope should be analyzed by immunoblotting. If analysis is done under non-reducing and reducing conditions and development is with a specific antibody against the displayed protein and gp64, then 4 identical gels/blots are required. Non-reducing conditions allow for detection of multimeric forms of the displayed protein, while reducing conditions offer the possibility to assess the portion of proteinX-gp64-fusion-protein in comparison to native gp64 (can be 50 %).

1. Denature $2 \times 10^6$ pfu A4p virus preparation (Subheading 3.3.5) per slot with sample buffer for 5 min at 95 °C. If you want to quantitatively compare vectors, then make certain that equal amounts of virus protein are loaded onto the gel.
2. Load onto SDS polyacrylamide gels (8 %) under non-reducing or reducing conditions (sample buffer + 1 mM DTT) and separate at 150 V.
3. Wash Gel in transfer buffer for 5 min.
4. Transfer onto Hybond-C extra membranes at 0.85 mA/cm$^2$.
5. Block membrane for 90 min with blocking buffer.
6. Incubate membrane with primary antibody, e.g., *Ac*V5 against gp64 in a 1:1000 dilution and/or your displayed protein in blocking buffer overnight.
7. Wash membrane with blocking buffer and detect primary antibodies with respective horseradish peroxidase conjugated antisera (dilution 1:1000, for 30 min).
8. Carefully wash membrane 5 times with PBS and visualize bands by the ECL detection system.

#### 3.4.3 Electron Microscopy

1. Prepare serial dilutions of the A4p virus preparation.
2. Float virus dilutions from **step 1** on carbon coated grids. This is done by simply adding a drop of the virus solution on the grid for at least 1 min and then excess virus solution is drained off by touching the edge of the grid to a piece of clean filter paper. The same procedure is performed with antibodies.
3. Expose with monoclonal primary antibody in 10 % (w/v) BSA. Use concentration recommended for immunofluorescence studies.
4. Wash grids 3 times with PBS and incubate with a 1:30 solution of 12 nm colloidal Gold-AffiniPure goat anti-mouse IgG in 10 %w/v BSA.
5. Wash grids 5 times with PBS.
6. Stain preparations with PTA and analyze with an electron microscope (*see* **Note 7**).

### 3.5 Gene Delivery into Mammalian Cells

Baculovirus-mediated gene transfer was initially described to be highly efficient into hepatocytes [1, 16]. Gene delivery and expression in mammalian cells depends on the promoter used, the not-yet known receptor on the surface and differences [17] in the ability of cells to differentially repress transgene expression.

#### 3.5.1 Transduction Procedure

1. Seed mammalian cells at a density of $3 \times 10^4$ cells/cm$^2$ (can be done from 96-well plate to big T-flask) so that they are 50–70 % confluent the next day. Untreated FCS or sera from other sources contains active complement, which inactivates the virus. It must be heat-inactivated for 30 min at 56 °C before adding to the cell culture medium.
2. The next day, remove medium and replace with fresh medium including the A4p virus preparation (Subheading 3.3.5) at a volume of 100 µL/cm$^2$. Incubate at 37 °C for 1 h. An MOI of 100 is a good starting concentration if no further information on the cell line is available. This MOI generates a 50 % transduction efficiency in the hepatocarcinoma cell line HuH7, which can be used as a control (*see* **Note 8**).
3. Remove virus inoculum from cells after 1 h and add fresh medium.
4. Analyze efficiency of gene delivery after 36 h (peak with a recombinant luciferase expression baculovirus in Huh7 cells [1]).

#### 3.5.2 Analysis of Gene Delivery

Analysis of gene delivery is dependent on the inserted mammalian expression cassette and based on numerous options. This issue is not discussed with a specific example. In order to experience this system, reporter gene expression cassettes, e.g., ß-galactosidase, GFP,

or luciferase, under control of a mammalian cell active promoter should be inserted into the baculoviral genome. Standard analytical methods are available in most labs (e.g., *see* Chapter 22).

## 4 Notes

1. This plasmid (pBacsurf_mam) is the basis of the reference baculovirus vector with wild-type envelope. Always generate this virus according to the procedure given under Subheading 3.3.1 for comparative studies with your envelope-modified variant.
2. Inserts must lack an internal stop codon and maintain the appropriate open reading frame. Select a unique cloning site based on the sites of pBACsurf_mam. If all three sites, i.e., *Sma*I, *Kpn*I, and *Pst*I, are within your mammalian expression cassette, then develop a strategy to first insert the PCR-fragment for envelope modification and then the mammalian expression cassette via *Eco*RV.
3. This procedure can be scaled down by at least a factor of 5 to save material and money. If you decide to scale down transfection, then use 24-well plates for cell culture and 96-well Polystyrol plates with round bottoms for the Plasmid/BaculoGold–Lipofectamine mixture.
4. At these steps, we recommend to prepare backups, which can be thawed and used in case a later procedure fails. It also helps retrospectively to define the step that led to an eventual discrepancy between your plasmid construct and virus preparation in the course of virus analysis (Subheading 3.4).
5. Virus titer peaks normally at about 24–36 h. Longer cultivation ends up in a lot of cell debris that is more difficult to purify later.
6. Although the use of unpurified viruses is described in the literature for gene delivery into mammalian cells, this step avoids potential pseudotransduction.
7. A variety of publications describe that gp64 and gp64-fusion proteins are localized at the poles of the virions. By electron microscopy, we mainly detected viruses that displayed either gp64 or fusion-proteins completely surrounding their loose-fitting envelope.
8. Addition of butyrate at a concentration of 1–10 mM or trichostatin at a concentration of 1 μM to the cells prior to the transduction procedure de-represses transgene expression and leads in most cells to increased infection efficiencies—the compounds are, however, to a certain extent toxic to the cells.

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