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

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### Summary

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 techniques are summarized and the reader is directed to detailed information found elsewhere in this book. Furthermore, many useful tips and the author's 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<sub>2</sub> (owing 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.

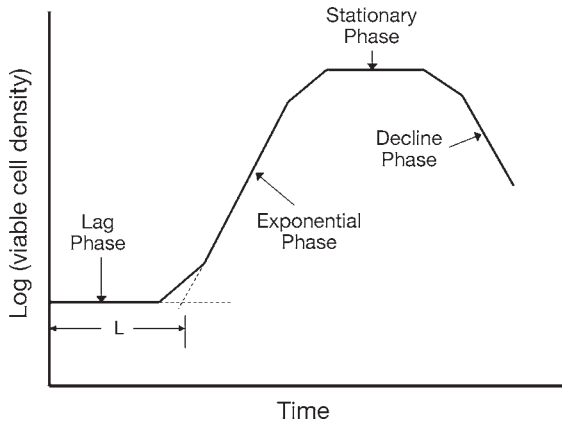


Fig. 1. A typical cell growth curve (cell density vs time) showing the lag, exponential, stationary, and decline phases. The length of the lag phase ( $L$ ) can be found by extending the lag and exponential phase lines and finding the time at which they intersect (as shown).

Following subculture, insect cells in batch culture (*see Note 2*) proceed through the typical growth phases (*see ref. 1, 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 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 ( $\mu$ ), 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 [DO] concentration, nutrient concentrations, temperature, pH, and so on). Furthermore, percentage cell viability is a measure of cell quality. Typical ranges of  $\mu$  and PDT are 0.029 to 0.035/h and 20 to 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 in **Subheading 3.1**.

Cell growth during the exponential growth phase can be represented by

$$N = N_0 \exp(\mu t), \quad (1)$$

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 ( $\text{h}^{-1}$ ), and  $t$  = time in culture (h).

## 1.2. Baculovirus Infection

Insect cells are infected with either a recombinant baculovirus (see Chapters 4 and 10) to produce a recombinant protein or with a wild-type baculovirus to produce biopesticides (recombinant baculovirus can also be used as biopesticides, see Chapters 18 and 20). 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 in **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 in **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 is 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 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 in **Subheading 3.3**.

## 2. Materials

### 2.1. Cell Growth

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

### 2.3. Cell Metabolism

1. DO monitor, e.g., YSI 5300A Biological Oxygen Monitor with microoxygen chamber (YSI Incorporated, Yellow Springs, OH) (*see Note 9*).
2. Nutrient and byproduct monitor, e.g., BioProfile 400 analyzer (Nova Biomedical Corp., Waltham, MA) (*see Note 10*).
3. A high-performance liquid chromatograph for amino acid quantification.

## 3. Methods

### 3.1. Cell Growth

Methods involved in routine cell growth in monolayer (i.e., attached) cultures are given in Chapter 8, **Subheading 3.1.** and Chapter 9, **Subheading 3.1.** Methods involved in adapting cells to suspension growth, adapting cells to serum-free medium, routine shaker flask culture, and routine spinner flask culture are given in Chapter 8, **Subheadings 3.2–3.4, 3.5., and 3.6.,** respectively. Additional details about shaker flask culture are given in Chapter 11, **Subheading 3.1.1.** The author prefers 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 percentage viability can be determined with a hemacytometer as indicated in Chapter 11, **Subheading 3.1.2.** This involves counting the cells in the four  $1 \times 1$ -mm squares on the hemacytometer grid as indicated in **Fig. 2.** This method utilizes the Trypan blue dye method for determining cell viability. The 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. Although Trypan blue dye exclusion is

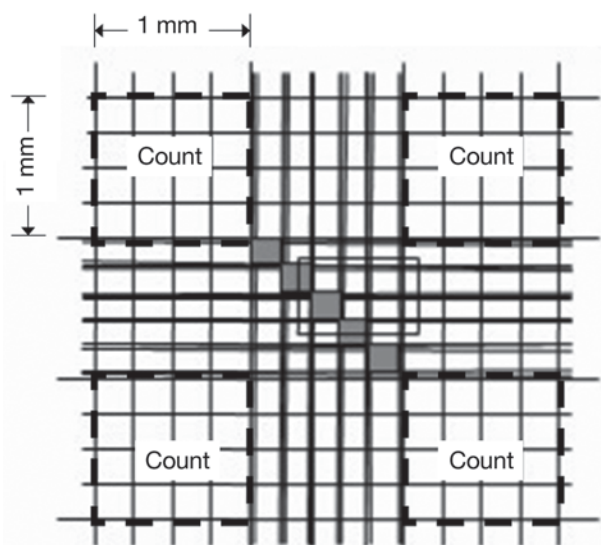


Fig. 2. Schematic of the hemacytometer grid used to count cells. Typically, the cells in the four corners (designated by “count”) are counted. Each of these regions has an area of 1 mm<sup>2</sup> and a depth of 0.1 mm; thus, the corresponding area is 0.1 mm<sup>3</sup>.

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 as a result of its simplicity and the fact that results can be obtained in approx 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 (Vanassas, VA, <http://www.atcc.org>).

The author prefers using either a Coulter particle counter or a Vi-CELL to determine cell density (*see Note 8* in this chapter and *Notes 5* and *7* in Chapter 11). Furthermore, the Trypan blue dye exclusion method can be used to determine cell viability if a Coulter particle counter is used. Note that the Vi-CELL determines both the cell density and viability. (Unfortunately, these are relatively expensive pieces of equipment that may be beyond the financial means of some laboratories.) If the Trypan blue dye exclusion method is used, then viable cell density is determined by multiplying the total cell density by the fraction of viable cells. Manufacturer instructions can be followed to deter-

mine cell densities, mean cell size, cell distribution, and cell viability (Vi-CELL only) with the Coulter particle counter or Vi-CELL.

Conducting cell counts in suspension cultures (i.e., spinner flasks, shaker flasks, 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* Chapter 9, **Subheading 3.1.**). 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**).

### 3.1.2. Evaluating $\mu$ , 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) vs 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 semi-log 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) vs 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**:

$$\ln(N) = \ln(N_0) + \mu t \quad (2)$$

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

6. Calculate the PDT as follows:

$$\text{PDT} = \frac{\ln(2)}{\mu} \quad (3)$$

For our example,  $\text{PDT} = \ln(2)/(0.0325/\text{h}) = 21.3 \text{ h}$ .

**Table 1**  
**Cell Density vs 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	$4.47 \times 10^6$

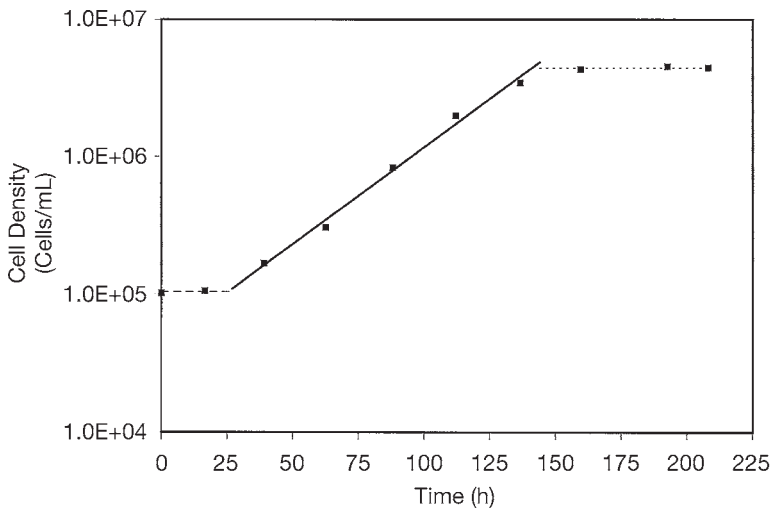


Fig. 3. Plot of growth curve data (Sf-9 cells grown in serum-free medium) to find the location of the exponential growth phase and the length of the lag phase (see Fig. 1).

- 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 is approx 27 h (Fig. 3).
- 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 previous text, but are repeated here for easy reference and to emphasize their importance.

- 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. It is recommended that the use of detergents be minimized if not totally eliminated.

2. The author prefers 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, the author has 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. The author has found that agitation become “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), DO limitations, 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* Chapter 15, **Note 4**). There seems to be some controversy over this issue; therefore, it is suggested that cell growth is compared with and without heat inactivation if FBS is used.
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, the author recommends freezing many vials of your cell culture that can be thawed periodically for consistent cell behavior in a series of experiments (*see* in Chapter 8, **Subheading 3.7.**; Chapter 9, **Subheadings 3.3.2.1.** and **3.3.2.2.**; and Chapter 16, **Subheading 3.2.**).
8. Insect cells are not as shear sensitive as commonly reported. For example, the author has found that increased agitation rates in bioreactors do not result in cell damage in the absence of bubble incorporation through cavitation, and so on (*see* Chapter 14, **Note 5**). 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 phase). The author has found that cell properties can change as cells are allowed to remain in stationary phase for an extended time. The author recommends 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). The author recommends  $5 \times 10^5$  cells/mL as a reasonable target seeding density.
11. Do not use antibiotics in routine cell culture (*see* Chapter 9, **Subheading 3.2.1.**). 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 baculovirus expression vector system 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 Invitrogen (Carlsbad, CA).

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 in Chapter 4, **Subheading 3.3.**

### *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, although an excellent cell line for producing recombinant proteins, is not as well suited to producing baculovirus stocks) and that these cells are infected at a relatively low MOI (approx 0.1) to prevent mutant accumulation (Chapter 9, **Subheading 3.4.1.**). Furthermore, it has been found that the addition of FBS (5–10%) significantly enhances the stability of baculovirus stocks stored at 4°C. Under these conditions the baculovirus is stable for up to approx 1 yr. Alternatively, baculovirus stocks can be stored at –85°C for long periods (*see* Chapter 9, **Subheading 3.4.2.**).

### *3.2.3. Determining Baculovirus Titer*

It is critical that an accurate (*see* **Note 13**) baculovirus titer (plaque forming units/milliliter [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 in Chapter 4, **Subheading 3.5**. The basic end point dilution procedure is described in Chapter 10, **Subheading 3.4**. 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 (Chapter 5, **Subheading 3.7.**) and the green fluorescent protein (GFP) (Chapter 21, **Subheading 3.2.**). 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 in Chapter 21, **Subheading 3.1.**) following baculovirus infection. Another method of titering baculovirus is through flow cytometry following binding of a fluorescent dye to baculovirus DNA (Chapter 11, **Subheading 3.2.3.**). 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 titering methods previously described 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 3.2.5.*).

#### 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

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

where where  $F(n, \text{MOI})$  is the fraction of cells infected with  $n$  baculovirus particles at a given MOI.

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:

$$\left( \begin{array}{l} \text{Fraction of insect cells} \\ \text{infected with Baculovirus} \\ \text{at a given MOI} \end{array} \right) = 1 - \exp(-\text{MOI}) \quad (5)$$

### 3.2.5. Infection of Insect Cell Cultures

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.

#### 3.2.5.1. CELL LINE SELECTION

Sf-9 and Tn-5 are the most common host cell lines used to express recombinant proteins with the baculovirus expression vector system. 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 Invitrogen (*see Note 14*) is a good choice if the recombinant protein is glycosylated. The reader is also referred to Chapters 6 and 7 regarding the wide range of available insect cell lines and methods to develop new cell lines as well as Chapter 17 regarding cell line modification to improve protein processing. Finally, Chapter 8 can be consulted regarding selecting an appropriate serum-free medium (*see Note 15*).

#### 3.2.5.2. TIME OF INFECTION AND MOI

The time of infection (i.e., the cell density at infection) and the MOI are interrelated owing 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. The author recommends using an MOI of 10 and infecting the cells at a density of  $1.5$  to  $2.0 \times 10^6$  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 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 approx 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 as a result of using low MOIs.

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

### 3.2.5.3. 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 lysed 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 approx 80%, although this should be adapted as appropriate to each specific case.

### 3.2.6. *Baculovirus Infection: Useful Tips*

Some of these tips were discussed previously within the text, 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 (approx 0.1) when producing baculovirus stocks to prevent mutant accumulation (Chapter 9, **Subheading 3.4.1.**).
3. The stability of baculoviruses stored at 4°C can be increased significantly by adding 5–10% FBS. The addition of FBS to the insect cell cultures during the infection process, however, could complicate subsequent recombinant protein purification owing to the high concentration of proteins in FBS. Alternatively, the baculovirus stocks can be stably stored at –85°C for long term (Chapter 9, **Subheading 3.4.2.**).
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 400g for 10 min or 1000g for 5 min) 1–4 h following baculovirus infection (4 h is enough 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. The author 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 in **Subheading 3.3**.

7. It is tempting simply to use media with very high levels of nutrients to prevent nutrient depletion. This approach, however, does not seem to work. For example, increasing the initial glucose concentration beyond a certain level (the author cannot find any definitive evidence regarding the specific concentration) does not result in increased maximum cell density prior to glucose depletion. This may be a result of 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. It is suspected (although the author 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

#### 3.3.1.1. USING A DO ELECTRODE

1. If a DO electrode is being used (e.g., in a bioreactor), then turn off the oxygen supply and record the 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) vs 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 in **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 percentage air saturation be converted to DO concentration in terms of moles per liter ( $M$ ). The solubility of oxygen in pure water when air is in the gas phase at 27°C is approx 0.26 mM (7). Thus, 100% air saturation corresponds to approx 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:

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

#### 3.3.1.2. USING A 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 3.3.1.1**.) because the cells will be discarded when the test is completed and it does not matter if cell metabolism is affected.
4. Follow **steps 2–5** given in **Subheading 3.3.1.1**. to determine the specific OUR.

### 3.3.2. Specific Nutrient Utilization and Byproduct Accumulation Rates

As shown next, 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).

**Table 2**  
**Cell Density and Glucose Concentration as a Function of Time in Sf-9 Cell Culture<sup>a</sup>**

Time postinfection (h)	Viable cell density (cells/mL)	Glucose concentration (mM)
-72	$2.2 \times 10^5$	48.0
-48	$4.8 \times 10^5$	45.5
-24	$1.0 \times 10^6$	39.5
0	$2.1 \times 10^6$	32.0
24	$2.3 \times 10^6$	21.5
48	$2.1 \times 10^6$	11.5
72	$2.2 \times 10^6$	5.5

<sup>a</sup>The cells were allowed to grow 72 h prior to baculovirus infection at an MOI of 10.

### 3.3.2.1. SPECIFIC RATES DURING EXPONENTIAL GROWTH PHASE

1. Determine the viable cell density (**Subheading 3.1.1.**) and nutrient (e.g., glucose) 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) vs 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  $\ln(\text{cell density})$  vs time yields a specific growth rate ( $\mu$ ) of 0.0313/h for the exponential phase ( $-72$  to 0 h pi). This follows the procedure given in **Subheading 3.1.2.** and **Fig. 3**.
  - b. Plot the cell density vs 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  $-1.16 \times 10^5$  cells/(mL-mM).
  - c. Calculate the specific utilization rate of glucose as follows:

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

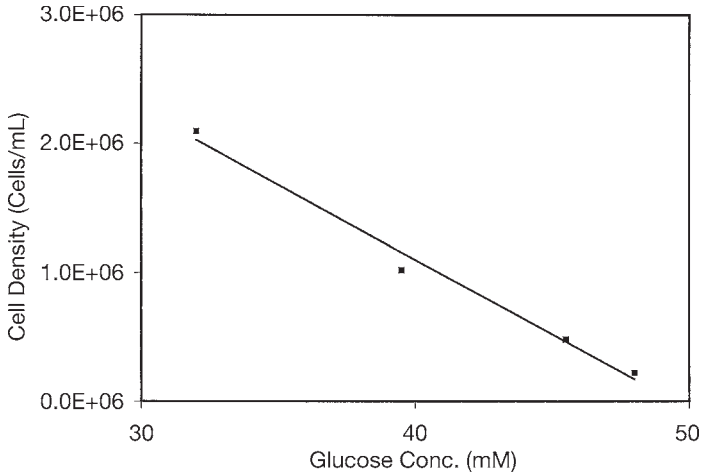


Fig. 4. Plot of cell density vs glucose concentration in the exponential phase for Sf-9 cell culture in serum-free medium. The slope of this curve is used to determine the specific glucose consumption rate during exponential phase.

### 3.3.2.2. 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 (the author recommends at least three to four data points with at least 6–8 h between them) (*see Note 20*).
3. Plot the component concentration (y-axis) vs time and determine the slope.
4. 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 four postinfection data points is  $2.2 \times 10^6$  cells/mL.
  - a. Plot the glucose concentration vs time as given in **Fig. 5**. For our example the slope is  $-0.373$  mM/h.
  - b. Calculate the specific glucose utilization rate as follows:

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

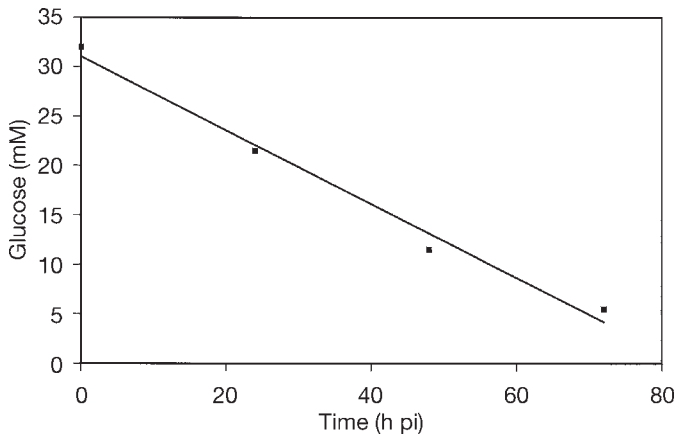


Fig. 5. Plot of glucose concentration vs time following baculovirus infection (multiplicity of infection = 10) of Sf-9 cell culture in serum-free medium at a constant cell density of approx  $2.2 \times 10^6$  cells/mL. The slope of this curve is used to determine the specific glucose consumption rate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

#### 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 that 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. Although the author prefers 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. The author has 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. The author prefers the term “exponential growth” because 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, the author has 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 or glutamine) or accumulation of toxic byproduct(s) (e.g., lactate 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 or Vi-CELL is optional since cell counts can also be obtained through the use of a hemacytometer. However, the author prefers using the particle counter to obtain total cell counts (note that this instrument 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.) The author has 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. Finally, the Vi-CELL, which can provide the cell density, cell size distribution, *and* cell viability, is a relatively recent advancement that is the author’s method of choice.
9. This oxygen monitor is applicable for small-scale cultures, e.g., shaker flasks. If a DO electrode is being used (e.g., in a bioreactor), then the electrode should be used as described in **Subheading 3.3**.
10. This bioprocess monitor will quantify glucose, lactate, glutamine, glutamate, ammonium, DO, 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 because cell growth is significantly disrupted by the cell detachment process.
12. In our example the long lag phase was probably a result of seeding at too low of a cell density ( $\sim 10^5$  cells/mL, see **Table 1**) in serum-free medium. The author recommends 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., 10-fold dilutions are used) and therefore the errors on a linear scale may be large. The author believes 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 analyzing the cell growth data 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:  $\alpha$ 2,6-sialyltransferase,  $\alpha$ 2,3-sialyltransferase,  $\beta$ 4-galactosyltransferase, *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 17 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, because serum contains a large amount 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. One hundred percent air saturation is the concentration of DO in solution when the solution is in equilibrium with an air (which contains 21 *M* percentage 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 approximately five (actually 100/21).
18. It is strongly recommended that the DO concentration not be allowed to go below 20% air saturation when measuring the oxygen utilization rate to prevent altering cell metabolism.
19. Determining the oxygen utilization rate by monitoring the DO 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). The author recommends the latter approach.
20. Many of the nutrients and byproducts can be determined using a bioprofile monitor by following the manufacturer directions. Furthermore, a high-performance liquid chromatography can be used to determine amino acid concentrations as described in **ref. 2**.

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## Introduction to Baculovirus Molecular Biology

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### Summary

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 yr 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, disulfide 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 thing of the past. Using baculoviruses as expression vectors is no longer the preserve of specialist virologists. 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 anti-foam 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. Although 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 a circular double stranded genome. They are characterized by their ability to occlude virions in a crystalline protein matrix. The family is divided into two genera, the granuloviruses (GVs) and the nucleopolyhedroviruses (NPVs). **Figure 1** illustrates an example of each of these genera. 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**). GV's produce small occlusion bodies (OBs) (0.16–0.30  $\mu\text{m} \times 0.30$ –0.50  $\mu\text{m}$ ) normally containing one or occasionally two virions encapsulated in a protein called granulin. NPVs produce larger OBs (0.5–1.5  $\mu\text{m}$  in diameter) composed of polyhedrin protein, which contain many virions. Infection by baculoviruses is limited to arthropods, with GV's having been isolated solely from lepidopteran species (butterflies and moths). The NPVs pathogenic for members of the order Lepidoptera have been subdivided into groups I and II based on molecular phylogenies (**1,2**). Some NPVs infectious for Diptera (flies), Hymenoptera (sawflies), and Trichoptera (caddis flies) have also been identified.

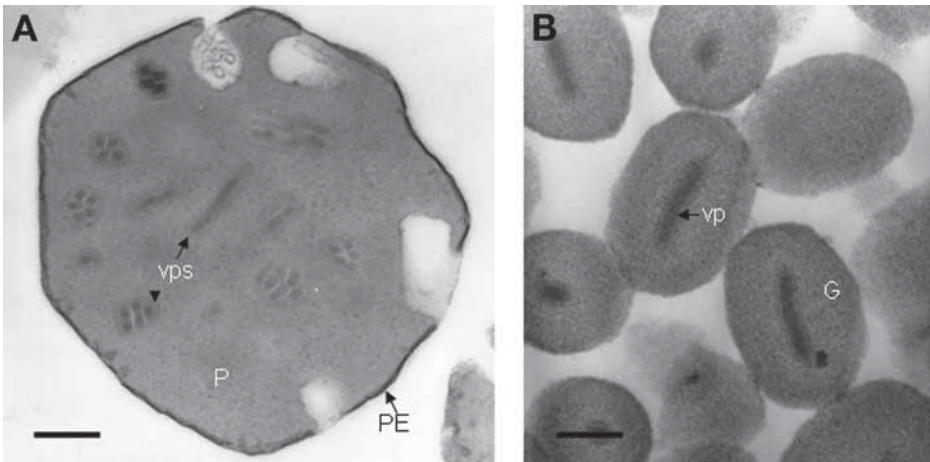


Fig. 1. Electron micrographs of *Autographa californica* nucleopolyhedrovirus (AcMNPV) polyhedron (A) and *Plodia interpunctella* granulovirus (PiGV) granules (B). Note the multiple virus particles (vps) in longitudinal or cross section in AcMNPV, whereas in PiGV each granule contains a single virus particle (vp). Virus particles are occluded within polyhedrin protein (P) or granulin (G). Polyhedra are bounded by an envelope (PE), which contains protein and carbohydrate. Bar = 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 (3). 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 (4).

There are two types of baculovirus progeny, budded virus (BV) and occlusion-derived virus (ODV), which are genetically identical (5). However, there are differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity (6–10).

BV particles possess spike-like structures known as peplomers, composed of the glycoprotein GP64 for group I NPVs, at one end of the virion (11). The GP64 protein is incorporated throughout the virus envelope, albeit at lower concentrations than at the peplomers (11). During infection, GP64 localizes to discrete areas of the plasma membrane at which points budding of virions takes place (12). Thus, as BV particles bud from the plasma membrane they acquire a plasma membrane-derived envelope containing the GP64 glycoprotein.

GVs and group II NPVs lack GP64 but instead encode homolog of another envelope protein, LD130 (13). Although the LD130-encoding baculoviruses do not contain *gp64* homolog, those encoding GP64 also encode a homolog 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 to the GP64 protein (14).

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 (15,16). Exposure 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 open reading frame (ORF) 119 of AcMNPV (17). A third gene product inferred to be a structural protein is encoded *pif-2*, which was identified in *Spodoptera exigua* NPV and is present in AcMNPV as ORF22 (18).

Other ODV-specific envelope proteins include ODV-E25 (19), ODV-E66 (20), ODV-E56 (21), ODV-E18, and ODV-E35 (22). These proteins, along with P74, have been found to associate with intranuclear microvesicle structures, which appear in the nucleus during infection (22,23). This association has led to the suggestion that these microvesicles play a role in ODV envelopment/envelopment (20,21). Observed invagination of the inner nuclear membrane during baculovirus infection has raised the possibility that it may act as the source of the microvesicles (24–26).

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 (27). 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 (28). 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 (22). This protein may be a cyclin homolog, involved in host cell cycle arrest during baculovirus infection (29). An envelope protein of both BV and ODV has also been identified (BV/ODV-E26) and found to be associated with intranuclear microvesicles (30). A seminal study of protein composition of ODV was conducted by using a combination of techniques (31). Screening expression libraries with antibodies generated to ODV or BV and mass spectroscopic analysis of ODV protein extracts identified up to 44 potential components unique to ODV.

The major component of the NPV occlusion body is the 29-kDa polyhedrin protein. Although 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 (32). Another region of the protein (amino acids 19–110) is required for assembly into large occlusion-like particles (32). 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 (33).

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 (34); however, a 34-kDa phosphorylated protein (PP34) has since been found to be covalently bound to the PE of AcMNPV via a thiol linkage (35). The PE is thought to increase the stability of the occlusion body in the environment and has been found to protect OBs from physical stress (35,36). A recombinant virus unable to produce PP34 was found to be deficient in PE formation and electron dense spacers, which are thought to be precursors of the envelope structure (36,37).

Both electron dense spacers and the PE have been found in association with fibrillar structures composed of the P10 protein (36,38). This protein, like polyhedrin, is produced in large quantities during the occlusion phase. The association of the PE with the P10-containing fibrillar structures suggests a role for the protein in assembly and proper association of the PE around the occlusion body matrix (38). Comparative analysis of P10 protein sequences revealed that they appear to have an amphipathic  $\alpha$ -helical terminus that condenses as coiled-coil multimers (39). 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. ODV initiates the primary infection in the gut epithelial cells, whereas BV is responsible for secondary rounds of infection in the insect host. Infection begins with ingestion of OBs by a suitable insect host larval stage, followed by dissolution of the occlusion body matrix in the alkaline midgut. ODV 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 (40). Removal of P74 or PIF1 or PIF2 virus particles by deletion of the appropriate gene has been demonstrated to prevent infection of insect larvae via the oral route (15–18). Midgut regenerative cells have also been found to be sites of primary infection, albeit at a lower frequency than columnar cells (41). 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 (42). The insect tracheal system has been identified as the main route used by the virus to spread from one tissue to another (43).

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) (44), and is thought to be the site of viral DNA replication and late gene transcription, as well as nucleocapsid assembly (45). 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 (12). 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 (13), which associates with BV particles, and the plasma membrane of infected cells, at similar positions to GP64 in group I NPV-infected cells (14). These F proteins are functionally analogous to GP64 because they can restore infectivity to mutant viruses lacking *gp64* (46). GP64 is essential for cell-to-cell BV transmission, because AcMNPV particles containing the protein, but not those lacking the protein, are able to disseminate virus to other cells (47).

Later in infection (approx 24 h pi), BV production is reduced and nucleocapsids are transported to sites of intranuclear envelopment and incorporation into OBs (36). 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, suggesting they may be the source of the envelope (20,22,23).

OBs accumulate until the terminal stages of infection, when the insect liquefies, releasing them into the environment. Liquefaction appears to be caused

by the production of both virus-encoded chitinase and cathepsin genes (48–50) and may also involve the *fp25k* product (51). 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 (52). 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 (53). This enhanced locomotory activity may well be virus-induced, because insects infected with a *B. mori* NPV mutant lacking the protein tyrosine phosphatase gene showed dramatically reduced enhanced locomotory activity before death at about 5 d (54). OBs 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 (33).

## 5. Baculovirus Gene Expression and Replication

The complete sequence of AcMNPV clone 6 has been determined. Analysis of the 133,894-bp genome suggested that the virus encodes 154 methionine-initiated, potentially expressed ORFs of 150 nucleotides or more (55). Other baculoviruses have also been sequenced. An up to date summary of completed genomes is available from NCBI. Baculovirus gene expression is divided into four temporal phases: immediate-early, delayed-early, late, and very late. 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. Although 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 (56).

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 (57,58). Only one transcript, which is that of the immediate-early gene *ie-1*, is known to be spliced (59). However, transcriptional units involving overlapping RNAs have been identified in the AcMNPV genome, which may provide an alternative means of introducing variety of expression (60,61). Overlapping transcripts composed of early and late RNAs with a common 3'-end have been identified in a number of regions (60,61). The *HindIII*-K fragment of the AcMNPV genome produces five over-

lapping RNAs, two immediate-early, one delayed-early, and two late gene transcripts, transcribed in the same direction and terminating at a common 3'-end (61). 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 (61).

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 (60–62). 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), also with a common 5'-end, most abundant at 24 h pi (62). 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 (62).

### 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 (63). 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  $\alpha$ -amanitin (64), 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 to 30 bases downstream (59,65–67). 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 (67–70). 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 (71).

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 (72). This sequence is also found at the start of *p143* (73). 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 (71).

Another *cis*-acting element identified in early promoters is a downstream activating region (DAR). A DAR has been identified in the 5' non-coding regions of the immediate-early *ie-1* gene (69). This element, located between positions +11 and +24, is necessary for optimal expression of *ie-1* early in infection (69). The core DAR sequence (A/T)CACNG has also been identified in the 5' non-coding region of the envelope glycoprotein encoding gene *gp64*, and has been found to stimulate the rate of early *gp64* transcription (69,74).

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 (66,67,75). The *ie-1* UAR has been found to increase promoter activity 2-fold early in Sf-21 cell infection (69), whereas the *p35* UAR was shown to be responsible for a 10- to 15-fold enhancement of basal transcription (66). 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 (66,67,76). 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* (66,67,73). 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* (74,77).

### 5.1.2. Transcriptional Enhancers

The AcMNPV genome contains homologous regions (*hrs*), rich in *EcoRI* sites, distributed throughout the genome (78). Eight of these regions (*hr1*, *hr1a*, *hr2*, *hr3*, *hr4a*, *hr4b*, *hr4c*, *hr5*) 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 *EcoRI* site and flanked on each side by direct repeats of about 20 bp (55,79). 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* (68,73,75,80–82). Promoter enhancement by *hrs* occurs in a position- and orientation-independent manner (81) and this enhancement is further augmented by the immediate-early *ie-1* (79), a viral transactivator

that has been found to bind to *hr* sequences (82–84). The *hr* 28-mer is the minimal sequence required for IE-1 mediated promoter enhancement (80–82). 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 (81–84). 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 (83,85).

### 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 (80). As well as stimulating expression of genes such as *p35*, *p143*, and *39K* (73,75,80,86), IE-1 is capable of stimulating its own promoter (87). The N-terminal region of the 582 residue IE-1 protein has been found to contain a transactivation domain, whereas the C-terminal of the protein contains a DNA-binding domain (83). A small basic domain between residues 537 and 538 has been identified as a nuclear localization signal, which functions upon dimerization of IE-1 (85). Transcripts of *ie-1* give rise to both spliced and unspliced RNAs. Unspliced transcripts encode IE-1 itself, whereas spliced transcripts encode another immediate-early transregulator, IE-0, identical to IE-1 except for 54 additional amino acids at its N-terminus (59,87). Although IE-0 is expressed only during the early phase of infection, IE-1 RNA is expressed in both the early and late phases (59). Transient expression assays have shown IE-1 to have a negative regulatory effect on *ie-0* promoter expression, whereas IE-0 transactivates the *ie-1* promoter (87). 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 (88).

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 (73,89). 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 (68). Like IE-0 expression, IE-2 regulation has been shown to be downregulated by IE-1 (90). 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 (91).

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 (73). However, the delayed-early 39K promoter was not stimulated by PE-38 (73), suggesting that PE-38 has a restricted transactivation range compared to IE-1, which is capable of stimulating both promoters (73,80).

Transcripts of *ie-0*, *ie-2*, and *pe-38* are expressed during the early phase of infection, whereas *ie-1* RNAs are expressed during the early and late phases. The fact that IE-1 downregulates expression of both IE-0 and IE-2 in transient assays suggests it may function to shut off immediate-early gene expression during the late phase of infection (87,90), whereas IE-1 stimulation of its own promoter suggests it positively regulates its own expression during infection (87). Baculovirus *pe-38* is transactivated by IE-1 when both are transfected into mammalian BHK-21 cells (92).

## 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 (93,94). 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 24.

AcMNPV infection of Sf-21 cells induces apoptosis 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 (95). Expression of the AcMNPV anti-apoptotic p35 gene blocks apoptosis allowing replication of the virus to proceed (93). The specific factors that activate the cell death pathway in AcMNPV-infected Sf-21 cells are unclear. Although BV binding alone is not sufficient to induce apoptosis, transient expression of the immediate-early gene *ie-1* has been found to induce cell death in Sf-21 cells (96). 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 (97).

Although some induction of apoptosis occurs upon *ie-1* expression, viral DNA replication is required for the full apoptotic response (98). 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 (98). It is possible that DNA synthesis induces apoptosis indirectly by promoting the onset of late gene expres-

sion. 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 (93). Infection of Sf-21 cells with wild type AcMNPV causes transient plasma membrane blebbing at approx 12 h pi (93). A similar effect was observed with vAcAnh infection of Sf-21 cells. However, although this blebbing disappeared in the wild type infection, it was found to intensify with the mutant infection, resulting in disintegration of cells into apoptotic bodies (93). 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 (93). 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 (93). A deletion in the *p35* gene, located in the *EcoRI*-S fragment of the AcMNPV genome, was subsequently identified as being responsible for the annihilator mutant phenotype (93). 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 (63,66,75). 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  $\alpha$ , and ultraviolet light (100 nm). A *Cydia pomonella* granulovirus *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 (99).

A characteristic of all members of the IAP family is the presence of one to three copies of an imperfect 70 amino acid repeat called a Baculovirus IAP Repeat (BIR) at the N-terminus of the protein (94). 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

caspsases (101). The mechanism by which baculovirus IAPs block apoptosis is distinct from that of P35. Although P35 is capable of directly interacting with and inhibiting the active caspase, IAP acts upstream of this by inhibiting maturation of the procaspase (102).

### 5.3. Baculovirus Replication

The *hrs*, identified as enhancers of early gene expression, have also been proposed as origins of viral DNA replication (78). 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 transfected into Sf-21 cells (103). As with early promoter enhancement, a single 28-mer is sufficient to support plasmid replication (82). 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 present (104). An AcMNPV non-*hr* containing origin has also been identified in the *HindIII*-K fragment of the genome (103). 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 (103).

Six genes, encoding P143 (DNA helicase), DNA polymerase (105), IE-1, late expression factor (LEF)-1 (primase), LEF-2 (primase associated protein) (106), and LEF-3, (single-stranded DNA binding protein) (107,108) have been found to be essential for transient DNA replication (109). In addition, *lef-11* was reported to be essential for AcMNPV replication in Sf-9 cells (110). DNA-independent ATPase activity has been associated with the DNA helicase of *T. ni* granulovirus (111). Deletion of the DNA polymerase gene from the virus genome abrogates virus replication (112).

Genes encoding P35, IE-2, PE-38, and LEF-7 are thought to play a stimulatory role in DNA replication (109,113). The stimulatory effect of the anti-apoptotic *p35* gene in transient assays is thought to be largely as result of its role in preventing premature cell death, although there is evidence suggesting that P35 may also be involved in the regulation of early genes (86). The *pe-38* gene product has been seen to play a role in the activation of expression of the baculovirus helicase homolog P143, whereas IE-2 stimulates expression of *pe-38*, as well as *ie-1* (73,114). Stimulation of viral DNA replication by LEF-7 was observed (113) and this protein has been found to contain two single-stranded binding protein motifs.

Baculovirus DNA replication is associated with distinct foci in the nuclei of infected cells (115). It was found that IE-2, LEF-3, and a further protein thought to play a role in AcMNPV replication, termed DNA-binding protein, colocalized with centers of viral DNA replication within the nucleus (115).

The importance of LEF-3 in replication of DNA 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 (**116,117**). It has also been found to play a role in enhancing the strand displacement activity of DNA polymerase (**105**). Interaction between LEF-1 and LEF-2 has been observed, and is thought to be required for DNA replication to occur (**118,119**). The IE-1, LEF-3, and P143 products interact with DNA *in vivo*, as demonstrated by formaldehyde cross-linking studies (**120**). Deletion of very late factor 1 reduces DNA replication to a third of normal levels and no BV is produced (**121**).

#### **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 (**122**). This viral polymerase is  $\alpha$ -amanitin-resistant and unable to transcribe from early gene promoters (**123**). The products of four *lef* genes, *lef-8*, *lef-4*, *lef-9*, and *p47*, have been identified as components of the AcMNPV RNA polymerase (**122**). The LEF-4 has guanylyltransferase activity (**124**) and RNA 5' triphosphatase and nucleoside triphosphatase activity (**125**). 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 (**113,126,127**). 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 (**128**).

The viral RNA polymerase is thought to carry out both 5' methyl capping and 3' polyadenylation of late and very late transcripts (**124,125,129**). 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 (**122,125**). LEF-4 has also been found to possess an ATPase activity, although the function of this activity is unknown (**129**). Both LEF-8 and LEF-9 contain conserved motifs present in the large subunits of other DNA-directed RNA polymerases (**126,130**). The conserved motif of LEF-8 had been proposed as a putative catalytic site of the enzyme (**126**), however, *lef-8* mutagenesis studies have revealed that both termini of the protein are essential for its function (**131**). In addition, a host cell protein, designated polyhedrin promoter-binding protein, has been identified that binds to very late promoters and is required for expression of these genes (**132,133**). 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 required for transcription initiation by eukaryotic RNA polymerases I, II, and III (133).

#### 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 (3,4,134). 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 (135). Mutations in sequences surrounding TAAG have been shown to reduce transcription at the level of transcription initiation (136). 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 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 (136–139). Mutations upstream of the TAAG motifs of both very late promoters exert a mild effect on expression (137,140). Thus, the polyhedrin promoter consists of a 49-bp 5' untranslated region and 20 bp upstream of the transcription start site (137), whereas the *p10* promoter consists of a 70-bp 5' untranslated region and 30 bp upstream of the transcription start site (138,140). Polyhedrin and *p10* promoters appear to be regulated differently, with *p10* expression occurring earlier in infection and at lower levels than that of polyhedrin (141).

#### 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 (127,142–144). A subset of these genes is involved in plasmid DNA replication, whereas another four are thought to be subunits of the viral RNA polymerase, as previously outlined. 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 (113). 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 (145). Further genes involved in transient late gene expression have been identified (144). One of these genes, termed *orf41*, was found to be necessary for late gene expression, whereas *orf69* was found to play a stimulatory role (144). LEF-12 is dispensable for

virus replication (146). It was suggested that IE0 could be regarded as the 20th LEF as it can replace IE1 in a transient replication assay (147).

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 (148). 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 (141).

The very late expression factor (*vlf*)-1, is specific for regulation of very late transcripts (149). 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 (150). 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 (151,152). Accelerated production of VLF-1 results in premature polyhedrin synthesis, showing the timing of VLF-1 expression to be important in very late gene transcription (152).

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 concatameric products of the DNA replication process (151). 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 (149). Viruses carrying *vlf*-1 null mutations have been found to be either non-viable or so defective in BV production that they were extremely difficult to propagate, indicating an essential role for the protein in baculovirus replication (151). 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 (152). Overexpressed and purified VLF1 added to transcription assays containing baculovirus RNA polymerase stimulated transcription of the polyhedrin gene promoter, but not 39K (153).

Serial passage of NPVs through cultured cell lines results in the appearance of a spontaneous mutant termed the "few polyhedra" (FP) mutant (154,155). With continued passage this FP phenotype becomes dominant (156). Fourteen passages of *T. ni* (Tn)MNPV in *T. ni* cells was found to result in a purely FP mutant population (156). 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 (155–157).

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 mod-

erately 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 analysis, in this region of the genome (158,159). 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 (160).

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 (161). Although identified as a structural protein of the nucleocapsids of BV and ODV (161), a large fraction of the protein remains associated with amorphous cytoplasmic bodies throughout infection (162).

Mutations in the *fp25k* gene alter the apparent expression or accumulation of several viral proteins. Rates of both polyhedrin biosynthesis and nuclear localization are reduced in cells infected with *fp25k* mutants (163). 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 (148). Although 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 (148). 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 BV is delivered to the haemocoel of the larval host (164–166). 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 made simultaneously in virus-infected cells (167–169). These vectors have been of particular use in assembling structures in insect cells that are com-

posed of more than one protein (170). Insect cells are also competent in accomplishing many of the posttranslational processes required when producing proteins, which have their origins in eukaryotic cells, so the products are normally biologically active.

The original method for producing recombinant baculoviruses required the native polyhedrin gene to be replaced 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. Although 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 (171).

## Acknowledgments

We thank Alex Patmanidi for providing electron micrographs of baculovirus occlusion bodies.

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## Baculovirus Transfer Vectors

Robert D. Possee and Linda A. King

### Summary

The production of a recombinant baculovirus expression vector normally involves mixing infectious virus DNA with a plasmid-based transfer vector and then cotransfecting 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. It is impractical to list every transfer vector that has ever been used. Instead, 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. A table listing the transfer vectors also included information on the parental virus that should be used with each one. Recent developments in recombinant baculovirus production are also described. Some of these permit 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

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

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 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 time-line of transfer vector developments. Instead, we will provide guidance on transfer vectors that can be used for all of the major applications of the baculovirus system. This chapter will focus 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. approx 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 have 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 cotransfect 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 cross-over 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 Subheading 1.2.*).

## **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 contains those vectors available for use with infectious virus DNA to make recombinant viruses after cotransfection 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 bod-

**Table 1**  
**Baculovirus Transfer Vectors and Parental Viruses**

Type ( <i>locus</i> )	Vector (size)	Promoter(s)	Features within cloning sites	Additional features/notes	Source	Parental virus (source)
Single promoter ( <i>polyhedrin</i> )	pFastBac <sup>TM</sup> 1 (4.8 kbp)	<i>Polyhedrin</i>	<i>Bam</i> HI, <i>Rsr</i> II, <i>Bss</i> HIII, <i>Eco</i> RI, <i>Stu</i> I, <i>Sal</i> I, <i>Sst</i> I, <i>Spe</i> I, <i>Not</i> I, <i>Nsp</i> V, <i>Xba</i> I, <i>Pst</i> I, <i>Xho</i> I, <i>Sph</i> I, <i>Kpn</i> I, <i>Hind</i> III		Invitrogen	Bac-to-Bac <sup>a</sup>
(Invitrogen)	pFastBacHT (4.8 kbp)		ATG, 6X His tag-TEV protease cleavage site, <i>Nhe</i> I, <i>Nco</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Stu</i> I, <i>Sal</i> I, <i>Sst</i> I, <i>Spe</i> I, <i>Not</i> I, <i>Nsp</i> V, <i>Xba</i> I, <i>Pst</i> I, <i>Xho</i> I, <i>Sph</i> I, <i>Kpn</i> I, <i>Hind</i> III			
Multiple promoter ( <i>polyhedrin</i> )	pFastBacDual (5.2 kbp)	<i>Polyhedrin</i>  <i>p10</i>	<i>Bam</i> HI, <i>Rsr</i> II, <i>Bss</i> HIII, <i>Eco</i> RI, <i>Stu</i> I, <i>Sal</i> I, <i>Sst</i> I, <i>Spe</i> I, <i>Not</i> I, <i>Nsp</i> V, <i>Xba</i> I, <i>Pst</i> I, <i>Hind</i> III  <i>Bbs</i> I, <i>Sma</i> I, <i>Xho</i> I, <i>Nco</i> I, <i>Nhe</i> I, <i>Pvu</i> II, <i>Nsi</i> I, <i>Sph</i> I, <i>Kpn</i> I	Dual expression from <i>polyhedrin</i> and <i>p10</i> gene promoters		
Single promoter ( <i>polyhedrin</i> )	pDEST <sup>TM</sup> 8 (6.5 kbp) PDEST10 (6.7 kbp) pDEST20 (7.0 kbp) pBlueBac4.5 (4.9 kbp)	<i>Polyhedrin</i>	Gateway <sup>®</sup> entry site  Gateway entry site N-term. 6X His tag Gateway entry site N-term. Glutathione-S-transferase (GST) <i>Nhe</i> I, <i>Bam</i> HI, <i>Sac</i> I, <i>Xho</i> I, <i>Bg</i> III, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Xba</i> I, <i>Eco</i> RI, <i>Bst</i> BI, <i>Hind</i> III, <i>Sal</i> I	Insert genes from Gateway entry vectors, such as pENTR <sup>TM</sup>      pBlueBac vectors contain 5'-end of <i>lacZ</i> to recombine with Bac-N-Blue virus DNA, which contains 3'-end of the <i>lacZ</i>		Bac-N-Blue virus DNA. Contains 3'-end of <i>lacZ</i> sequences for recombination with pBlueBac transfer vectors to produce full-length gene for blue color selec-
	pBlueBac-4.5V5-His (5.0 kbp)	<i>Polyhedrin</i>	<i>Nhe</i> I, <i>Bam</i> HI, <i>Xho</i> I, <i>Sac</i> I, <i>Bg</i> III, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Xba</i> I, <i>Eco</i> RI, <i>Bst</i> BI, <i>Hind</i> III, V5 epitope, AgeI, 6X His tag, Stop			

pBlueBac-4.5/V5-His-TOPO (5.0 kbp)	<i>Polyhedrin</i>	<i>NheI, BamHI, XhoI, SacI, BglII, PstI, KpnI, SmaI, XbaI, TOPO, BstBI, HindIII, V5 epitope, AgeI, 6X His tag-Stop</i>	TOPO site permits insertion of PCR products	tion in transfected insect cells. (Invitrogen)
pBlueBac-His2 A, B, C (4.9 kbp)	<i>Polyhedrin</i>	ATG-6X His tag-Xpress™ epitope-EK recognition site, <i>BamHI, SacI<sup>b</sup>, XhoI, BglII, PstI, KpnI, NcoI, EcoRI, BstBI, HindIII, Sall</i>	A, B, and C represent versions for three reading frames	
pMelBac A, B, C (4.8 kbp)	<i>Polyhedrin</i>	ATG, Honeybee melittin secretion signal, <i>BamHI, SacI<sup>b</sup>, XhoI, BglII, PstI, KpnI, NcoI, EcoRI, BstBI, HindIII, Sall</i>	A, B, and C represent versions for three reading frames. Honeybee melittin secretion signal	
pVL1392 (9.6 kbp)	<i>Polyhedrin</i>	<i>BglII, PstI, NotI, EagI, EcoRI, XbaI, SmaI, BamHI</i>		BD Biosciences Pharmingen AcMNPV (wildtype virus DNA)
pVL1393 (9.6kbp)	<i>Polyhedrin</i>	<i>BamHI, SmaI, XbaI, EcoRI, NotI, EagI, PstI, BglII</i>		BacPAK6 (13)
pAcG1 (8.5 kbp)	<i>Polyhedrin</i>	ATG, GST, <i>BamHI, SmaI, EcoRI</i>		BaculoGold (BD Biosciences Pharmingen)
pAcG2 (8.5 kbp)	<i>Polyhedrin</i>	ATG, GST, Thrombin cleavage- <i>BamHI, SmaI, EcoRI</i>		Bac1000
pAcG3X (8.5 kbp)	<i>Polyhedrin</i>	ATG, GST, Factor Xa cleavage, <i>BamHI, SmaI, EcoRI</i>		Bac2000
pAcGHLT-A, (8.7 kbp)	<i>Polyhedrin</i>	ATG, GST, <i>BamHI</i> -6X His tag, Protein kinase A site, Thrombin cleavage- <i>NdeI, EcoRI, StuI, NcoI, SacI, NotI, Sse8387I, PstI, KpnI, SmaI, BglII</i>		Bac3000
				The latter two virus DNAs contain deletions of AcMNPV genes. (Novagen)
				<i>flashBac™</i> Cloned version of AcMNPV unable to replicate in

(continued)

**Table 1 (Continued)**

Type (locus)	Vector (size)	Promoter(s)	Features within cloning sites	Additional features/notes	Source	Parental virus (source)
pAcGHLT-B, (8.7 kbp)	<i>Polyhedrin</i>		ATG, GST, <i>Bam</i> HI, 6X His tag-Protein kinase A site, Thrombin cleavage- <i>Xho</i> I, <i>Eco</i> RI, <i>Stu</i> I, <i>Nco</i> I, <i>Sac</i> I, <i>Not</i> I, <i>Sse</i> 8387I, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Bgl</i> II			insect cells unless rescued with appropriate transfer vector (Oxford Expression Technologies)
pAcGHLT-C, (8.7 kbp)	<i>Polyhedrin</i>		ATG, GST, <i>Bam</i> HI, 6X His tag-Protein kinase A site, Thrombin cleavage, <i>Nde</i> I, <i>Xho</i> I, <i>Eco</i> RI, <i>Stu</i> I, <i>Nco</i> I, <i>Sac</i> I, <i>Not</i> I, <i>Sse</i> 8387I, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Bgl</i> II			
pAcGP67-A (9.8 kbp)	<i>Polyhedrin</i>		ATG, GP64 signal sequence, <i>Bam</i> HI, <i>Sma</i> I, <i>Xba</i> I, <i>Eco</i> RI, <i>Not</i> I, <i>Eag</i> I, <i>Pst</i> I, <i>Bgl</i> III, <i>Ppum</i> I	A, B, and C represent three different reading frames and also contain slightly different restriction sites.		
pAcGP67-B (9.8 kbp)	<i>Polyhedrin</i>		ATG, GP64 signal sequence, <i>Bam</i> HI, <i>Sma</i> I, <i>Nco</i> I, <i>Eco</i> RI, <i>Not</i> I, <i>Eag</i> I, <i>Pst</i> I, <i>Bgl</i> III			
pAcGP67-C (9.8 kbp)	<i>Polyhedrin</i>		ATG, GP64 signal sequence, <i>Bam</i> HI, <i>Sma</i> I, <i>Nco</i> I, <i>Eco</i> RI, <i>Not</i> I, <i>Eag</i> I, <i>Pst</i> I, <i>Bgl</i> III, <i>Ppum</i> I			
pAcHLT-A (8.1 kbp)	<i>Polyhedrin</i>		ATG, GST, <i>Bam</i> HI, 6X His tag, Protein kinase A site-Thrombin cleavage, <i>Nde</i> I, <i>Eco</i> RI, <i>Stu</i> I, <i>Nco</i> I, <i>Sac</i> I, <i>Not</i> I, <i>Sse</i> 8387I, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Bgl</i> II	A, B, and C represent three different reading frames and also contain slightly different restriction sites.		
pAcHLT-B (8.1 kbp)	<i>Polyhedrin</i>		ATG, GST, <i>Bam</i> HI, 6X His tag, Protein kinase A site, Thrombin cleavage- <i>Xho</i> I, <i>Eco</i> RI, <i>Stu</i> I, <i>Nco</i> I, <i>Sac</i> I, <i>Not</i> I, <i>Sse</i> 8387I, <i>Pst</i> I, <i>Kpn</i> I, <i>Sma</i> I, <i>Bgl</i> II			

	pAcHLT-C (8.1 kbp)	<i>Polyhedrin</i>	ATG, GST, 6X His tag, Protein kinase A site, Thrombin cleavage site, <i>NdeI</i> , <i>XhoI</i> , <i>EcoRI</i> , <i>StuI</i> , <i>NcoI</i> , <i>SacI</i> , <i>NotI</i> , <i>Sse8387I</i> , <i>PstI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>BglII</i>	
	pAcSecG2T (8.6 kbp)	<i>Polyhedrin</i>	ATG, GP64 signal sequence, GST, Thrombin cleavage site, <i>BamHI</i> , <i>SmaI</i> , <i>EcoRI</i>	
	pAcSG2 (5.5 kbp)	<i>Polyhedrin</i>	<i>XhoI</i> , <i>EcoRI</i> , <i>StuI</i> , <i>NcoI</i> , ATG, <i>SacI</i> , <i>NotI</i> , <i>EagI</i> , <i>Sse8387I</i> , <i>PstI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>BglII</i>	ATG codon for fusion proteins
Single promoter ( <i>Basic/p6.9</i> )	pAcMP2 (9.8 kbp)	<i>Basic (p6.9)</i>	<i>PstI</i> , <i>NotI</i> , <i>EagI</i> , <i>EcoRI</i> , <i>XbaI</i> , <i>BamHI</i>	<i>p6.9</i> promoter provides late gene expression
	pAcMP3 (9.8 kbp)		<i>BamHI</i> , <i>XbaI</i> , <i>EcoRI</i> , <i>NotI</i> , <i>EagI</i> , <i>PstI</i> , <i>BglII</i>	
Multiple promoter ( <i>Polyhedrin</i> )	pAcUW21 (9.2 kbp)	<i>Polyhedrin</i> <i>p10</i>	See <b>Note</b> <i>PacI</i> , <i>BglII</i> , <i>EcoRI</i>	Polyhedrin gene intact; foreign gene expression from <i>p10</i> promoter
	pAcUW51 (5.8 kbp)	<i>Polyhedrin</i> <i>p10</i>	<i>BamHI</i> <i>BglII</i> , <i>EcoRI</i>	Dual expression vector from polyhedrin and <i>p10</i> promoters
	pAcAB3 (10.1 kbp)	<i>P10</i> <i>Polyhedrin</i> <i>p10</i>	<i>SmaI</i> , <i>BamHI</i> <i>XbaI</i> , <i>StuI</i> <i>BglII</i> , <i>EspI</i>	Triple expression vector from polyhedrin and 2X <i>p10</i> promoters
	pAcDB3 (6.0 kbp)	<i>P10</i> <i>Polyhedrin</i> <i>p10</i>	<i>SmaI</i> , <i>BamHI</i> <i>XbaI</i> , <i>StuI</i> <i>BglII</i> , <i>EcoRI</i>	Triple expression vector from polyhedrin and 2X <i>p10</i> promoters.
	pAcAB4 (10.2 kbp)	<i>Polyhedrin</i> <i>p10</i> <i>polyhedrin</i> <i>p10</i>	<i>BamHI</i> <i>SmaI</i> <i>XbaI</i> , <i>StuI</i> <i>BglII</i> , <i>EcoRI</i> , <i>EspI</i>	Smaller version of pAcAB3 Quadruple expression vector from 2X polyhedrin and 2X <i>p10</i> promoters

(continued)

**Table 1 (Continued)**

Type (locus)	Vector (size)	Promoter(s)	Features within cloning sites	Additional features/notes	Source	Parental virus (source)
Single promoter ( <i>Polyhedrin</i> )	pBAC-1 (5.3 kbp)	<i>Polyhedrin</i>	<i>Bam</i> HI, <i>Stu</i> I, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Ava</i> I, <i>Xho</i> I, 6X His tag, <i>Sty</i> I, <i>Avr</i> II, <i>Bpu</i> 1102, <i>Sph</i> I		Novagen	
	pBacgus-1 (7.4 kbp)	<i>Polyhedrin</i>	<i>Bam</i> HI, <i>Stu</i> I, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Ava</i> I, <i>Xho</i> I, 6X His tag, <i>Sty</i> I, <i>Avr</i> II, <i>Bpu</i> 1102, <i>Sph</i> I	$\beta$ -glucuronidase under <i>p6.9</i> promoter control to monitor recombinant virus production		
	pBAC-2cp (5.4 kbp)	<i>Polyhedrin</i>	<i>Nco</i> I, 6X His tag, <i>Sac</i> II, thrombin cleavage site, S tag, <i>Pf</i> MI, <i>Nhe</i> I, enterokinase cleavage site, LIC site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I, 6X His tag, <i>Avr</i> II, <i>Bpu</i> 1102I, <i>Sph</i> I	A Ligation-Independent Cloning (LIC) version of the vector is available for directional cloning PCR products.		
	pBacgus-2cp (7.6 kbp)	<i>Polyhedrin</i>	<i>Nco</i> I, 6X His tag, <i>Sac</i> II, thrombin cleavage site, S tag, <i>Pf</i> MI, <i>Nhe</i> I, enterokinase cleav- age site, LIC site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I, 6X His tag, <i>Avr</i> II, <i>Bpu</i> 1102I, <i>Sph</i> I	A LIC version of the vector is available for directional cloning PCR products. $\beta$ -glucuroni- dase under <i>p6.9</i> promoter control to monitor recombinant virus production		
	pBAC-3 (5.5 kbp)	<i>Polyhedrin</i>	Gp64 signal peptide, <i>Nco</i> I, 6X His tag, <i>Sac</i> II, thrombin cleavage site, S tag, <i>Pf</i> MI, <i>Nhe</i> I, entero- kinase cleavage site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I, 6X His tag, <i>Avr</i> II, <i>Bpu</i> 1102I, <i>Sph</i> I			

Multiple promoter ( <i>polyhedrin</i> )	pBAC4x-1 (5.9 kbp)	<i>P10</i> <i>Polyhedrin</i> <i>p10</i> <i>Polyhedrin</i>	<i>Bgl</i> II, <i>Eco</i> RI, <i>Bsu</i> 36I <i>Xba</i> I, <i>Stu</i> I <i>Sma</i> I, <i>Spe</i> I <i>Bam</i> HI, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I 6X His tag, <i>Sty</i> I, <i>Bpu</i> 1102I, <i>Sph</i> I	Quadruple expression vector from 2X <i>polyhedrin</i> and 2X <i>p10</i> promoters.
	pBACgus4x-1 (8.1 kbp)	<i>P10</i> <i>Polyhedrin</i> <i>p10</i> <i>Polyhedrin</i>	<i>Bgl</i> II, <i>Eco</i> RI, <i>Bsu</i> 36I <i>Xba</i> I, <i>Stu</i> I <i>Sma</i> I, <i>Spe</i> I <i>Bam</i> HI, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I 6X His tag, <i>Sty</i> I, <i>Bpu</i> 1102I, <i>Sph</i> I	Quadruple expression vector from 2X <i>polyhedrin</i> and 2X <i>p10</i> promoters. $\beta$ -glucuronidase under <i>p6.9</i> promoter control to monitor recombinant virus production.
Single promoter ( <i>polyhedrin</i> )	pBAC-5 (5.5 kbp)	<i>Gp64</i>	<i>Nco</i> I, 6X His tag, <i>Sac</i> II, Thrombin cleavage site, S tag, <i>Pfl</i> MI <sup>c</sup> , <i>Nhe</i> I, enterokinase cleavage site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I, 6X His tag, <i>Avr</i> II, <i>Bpu</i> 1102I, <i>Sph</i> I	Has early/late <i>gp64</i> promoter but no signal peptide coding region.
	pBACgus-5 (7.7 kbp)	<i>Gp64</i>	<i>Nco</i> I, 6X His tag, <i>Sac</i> II, Thrombin cleavage site, S tag, <i>Pfl</i> MI <sup>c</sup> , <i>Nhe</i> I, enterokinase cleavage site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI, <i>Eco</i> RI, <i>Sac</i> I, <i>Hind</i> III, <i>Eag</i> I, <i>Not</i> I, <i>Xho</i> I, 6X His tag, <i>Avr</i> II, <i>Bpu</i> 1102I, <i>Sph</i> I	$\beta$ -glucuronidase under <i>p6.9</i> promoter control to monitor recombinant virus production. Has early/late <i>gp64</i> promoter but no signal peptide coding region
	pBAC-6 (5.6 kbp)	<i>Gp64</i>	GP64 signal sequence, <i>Nco</i> I, 6X His tag, <i>Sac</i> II, Thrombin cleavage site, S tag, <i>Pfl</i> MI <sup>c</sup> , <i>Nhe</i> I, enterokinase cleavage site, <i>Sma</i> I, <i>Srf</i> I, <i>Bse</i> RI, <i>Stu</i> I, <i>Bam</i> HI,	Has early/late <i>gp64</i> promoter and signal peptide coding region.

(continued)

Table 1 (Continued)

Type (locus)	Vector (size)	Promoter(s)	Features within cloning sites	Additional features/notes	Source	Parental virus (source)
			<i>EcoRI, SacI, HindIII, EagI, NotI, XhoI, 6X His tag, AvrII, Bpu1102I, SphI</i>			
	pBACgus-6 (7.7 kbp)	<i>Gp64</i>	GP64 signal sequence, <i>NcoI, 6X His tag, SacII, Thrombin cleavage site, S tag, PflMI<sup>c</sup>, NheI, enterokinase cleavage site, SmaI, SrfI, BseRI, StuI, BamHI, EcoRI, SacI, HindIII, EagI, NotI, XhoI, 6X His tag, AvrII, Bpu1102I, SphI.</i>	Has early/late gp64 promoter and signal peptide coding region. $\beta$ -glucuronidase under <i>p6.9</i> promoter control to monitor recombinant virus production.		
	pBACsurf-1 (9.4 kbp)	<i>Polyhedrin</i>	<i>SpeI, gp64 signal sequence, PstI, KpnI, SmaI, gp64 coding region</i>	Designed for incorporating target proteins on the virion surface by utilizing gp64 signal sequence and membrane anchor region.		
Multiple promoter ( <i>polyhedrin</i> )	pUCDM (3.0 kbp)	<i>Polyhedrin</i>  <i>p10</i>	<i>BamHI, RsrII, BssHIII, StuI, SalI, SacI, XbaI, PstI, BbsI, SmaI, XmaI, XhoI, NheI, NsiI, SphI</i>	<i>BstZ171, SpeI, ClaI, NruI</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 <i>AvrII</i> and <i>PmeI</i> sites, respectively.	(34)	DH10BMultiBac <sup>cre</sup> maintained in <i>E. coli</i> . Contains Tn7 site at the polyhedrin locus and Cre-loxP site in lieu of chitinase/cathepsin genes for simultaneous integration

Multiple promoter (chitinase/cathepsin) (5.3 kbp)	pFBDM	<i>Polyhedrin</i>	<i>Bam</i> HI, <i>Rsr</i> II, <i>Bss</i> HIII, <i>Eco</i> RI, <i>Stu</i> I, <i>Sal</i> I, <i>Sac</i> I, <i>Not</i> I, <i>Bst</i> BI, <i>Xba</i> I, <i>Pst</i> I, <i>Hind</i> III	Polyhedrin and p10 multicloning sites are flanked by <i>Avr</i> II and <i>Pme</i> I sites, respectively	of foreign genes. Cre-loxP integra- tion is mediated by pBADZ-His <sup>6</sup> Cre under arabinose control (34).
Single promoter ( <i>p10</i> )	pAcUW1 (4.6 kbp)	<i>p10</i>	<i>Bgl</i> II, <i>Hind</i> III	(30)	AcUW1.lacZ virus DNA linearized with <i>Bsu</i> 36I (30)
Multiple promoter ( <i>p10</i> )	pAcUW42 (7.1 kbp) pAcUW43 (7.1 kbp)	<i>p10</i> <i>Polyhedrin</i> <i>p10</i> <i>Polyhedrin</i>	<i>Bgl</i> II, <i>Pst</i> I, <i>Not</i> I, <i>Xba</i> I, <i>Kpn</i> I, <i>Sma</i> I <i>Bam</i> HI <i>Sma</i> I, <i>Kpn</i> I <i>Xba</i> I, <i>Not</i> I, <i>Pst</i> I, <i>Bgl</i> II <i>Bam</i> HI	BD Biosciences Pharming	

<sup>a</sup>Maintained in *E. coli*.

<sup>b</sup>Missing in C version.

<sup>c</sup>Within S tag.

ies 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 cotransfection experiment could be less than 0.1% (**11**). This required a very hard search for polyhedra-negative plaques after staining good quality titrations in insect cells. A good quality microscope and an experienced eye were needed. Screening for more than five recombinant viruses simultaneously was almost impossible because of the number of titrations required. This system was not amenable to nonspecialist use of baculovirus expression vectors.

### 1.2.2. Linear Virus DNA

The proportion of recombinant virus plaques that could be isolated from a cotransfection 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 *Bsu36I* 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  $\beta$ -galactosidase into the AcMNPV genome under the control of the polyhedrin gene promoter.

The AcMNPV genome was also modified to contain *Bsu36I* sites on both sides of the  $\beta$ -galactosidase sequences, so that digestion with this enzyme removed the gene and also part of a virus gene (open reading frame [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  $\beta$ -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**).

The defective linear parental baculovirus DNA concept was also exploited in a modification of a system originally devised for easy selection of recombinant viruses by coinserion of the  $\beta$ -galactosidase gene with the foreign gene of choice (**18**). The transfer vector contained the full-length  $\beta$ -galactosidase coding region under the control of early (*etl*) or very late (*p10*) gene promoters inserted upstream of the polyhedrin gene promoter and appropriate cloning

sites for foreign genes. Flanked by homologous sequences also present in the parental virus, this dual expression cassette is inserted into the virus genome after cotransfecting of insect cells with this transfer vector and linearized parental virus DNA.

The system was improved by inserting the 3'-end of the  $\beta$ -galactosidase coding region into a virus genome that could be linearized with *Bsu36I* to remove part of ORF1629 and render the DNA defective. Appropriate plasmid transfer vectors contain the full length ORF1629 and the 5'-end of  $\beta$ -galactosidase that overlaps the portion within the parental virus genome. These features flank the foreign gene to be inserted into the recombinant virus. Cotransfection of insect cells with digested virus DNA and the transfer vector rescues the defective genome and introduces the foreign gene and full length  $\beta$ -galactosidase sequences so that recombinant virus produce blue plaques in a titration in insect cells. This is a good example of how it is very important to use the correct plasmid transfer vector with the parental virus DNA. The pBlueBac transfer vectors will not work with alternative parental virus DNAs.

Although the use of AcMNPV DNA digested with *Bsu36I* 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 *Escherichia 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 automated methods. The virus DNA is now marketed as *flashBac*<sup>TM</sup> 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 trans-

position 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 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 *attR* 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  $\beta$ -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-h enzymatic reaction. The correct introduction of the foreign gene into the virus should be accompanied by removal of a  $\beta$ -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  $\beta$ -galactosidase production. Unlike the bacmid system previously described, 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 it to be activated. 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-nt region and a 50-nt downstream 5' non-coding 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' non-coding region that was suboptimal for very late gene expression. All vectors used currently have the complete 5' non-coding 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 postexpression 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 postinfection. 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 Pharmingen (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 posttranslational modifications are given in Chapter 17. 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 such as pBAC-5 and -6 for earlier expression of recombinant products (Table 1).

### 2.3. Transfer Vectors for Positive Selection of Recombinant Viruses

Some transfer vectors contain reporter genes that are coinserted within the virus genome and make it easier to identify the recombinant virus in a plaque assay. The pBlueBac series of transfer vectors from Invitrogen were designed for this purpose by incorporating the 5'-end of the *lacZ* gene so that it recombines with the 3'-end of the same gene in the parental virus. The pBacgus variants from Novagen have a copy of the  $\beta$ -glucuronidase coding region under the control of the basic protein or p6.9 gene promoter. Positive selection of viruses is also possible when using pAcUW21, which has a copy of the p10 promoter inserted upstream of the complete polyhedrin gene. When cotransfected with a polyhedrin-negative virus, recombinant plaques contain polyhedra and are easily scored. This vector is particularly useful for making recombinant viruses that express a gene encoding an insecticidal product intended for use as a modified bioinsecticide. See Chapter 18 for details about using baculoviruses as bioinsecticides.

### 2.4. 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 *Bsu36I* (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*III, was inserted *in lieu* of the p10 ATG and 5'-end of the coding region, but a *Hind*III 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 Pharmingen; [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 Tn7 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

Only two vectors are currently available for dual gene expression at the *p10* locus. Both pAcUW42 and 43 are developments of pAcUW1, but 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 are inserted between the two promoters to terminate transcripts from the *p10* promoter.

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

A few 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, pBAC-6, and pBACgus-6 from Novagen). The pBAC-3 vector uses the polyhedrin gene promoter but both pBAC-6 variants employ the native *gp64/67* promoter, which directs foreign gene expression in both early and late phases. Invitrogen markets pMelBacA, B, and C, which are three variants of a polyhedrin promoter-containing vector that incorporate the honeybee melittin secretion signal (35), accompanied by a multiple cloning site. Surface display of recombinant proteins on the budded virus particles is feasible if pBACsurf-1 (Novagen) 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. Conclusion

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 cotransfection 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 *flashBac*<sup>TM</sup> system, which utilizes a one-step cotransfection step in insect cells that can be automated. 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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## Recombinant Baculovirus Isolation

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### Summary

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<sup>®</sup> or BaculoDirect<sup>™</sup>, 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. Additionally, many systems require a plaque-assay to separate parental and recombinant virus prior to amplification and use of the recombinant virus. 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 (nonrecombinant) virus where this is necessary; methods for the small-scale amplification of recombinant virus; and subsequent titration by plaque-assay. 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; cotransfection; 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<sup>™</sup>, Invitrogen) now utilizes Gateway<sup>®</sup> 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 into the virus DNA, resulting in a recombinant virus genome. This process was first described by Smith et al. (1) in which they described the production of a recombinant virus expressing the  $\beta$ -interferon gene. The genome then replicates to produce recombinant virus (budded virus phenotype only, as the polyhedrin gene is no longer functional), which can be harvested from the culture medium for further propagation and analysis of foreign gene expression.

In most baculovirus expression systems available 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 this 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  $\beta$ -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  $\beta$ -galactosidase (2).

The efficiency with which recombinant virus could be recovered was improved by the addition of a unique restriction enzyme site (*Bsu36I*) 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% (2,3). Homologous recombination between the transfer vector and the linear DNA recircularized 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 *Bsu36I* 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 *Bsu36I* restriction enzyme sites in two flanking genes on either side of *lacZ*. Digestion with *Bsu36I* removes the *lacZ* gene and a fragment of an essential gene (open reading frame [ORF] 1629; [5]) producing linear BacPAK6 virus DNA that is unable to replicate within insect cells. Cotransfection of insect cells with BacPAK6 DNA and a transfer vector containing the gene of interest, under the control of the polyhedrin gene promoter, restores ORF1629 and recircularizes the virus DNA by allelic replacement. The recombinant baculovirus DNA is then able to replicate in insect cells and in the late phase of infection, virions are assembled and recombinant baculoviruses are produced. However, *Bsu36I* 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 (Invitrogen), 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-*attTn7* target site in the bacmid DNA and a mini-Tn7 element in the donor plasmid. Recombinant bacmid-containing colonies are obtained by plating onto selective media, before 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 3.7.) and the resulting DNA is 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 at all (neither in bacterial cells nor insect cells) (9–11). This technology has been commercialized as *flashBAC*™ (Oxford Expression Tech-

nologies [www.expressiontechnologies.com]). The *flashBAC* 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, 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 (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 (Invitrogen [www.invitrogen.com]). 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 *attR* 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, using standard protocols such as the one described in **Subheading 3.1.**

The following protocols cover the essential stages in making and selecting recombinant baculoviruses. In **Subheading 3.1.**, the authors describe a generic protocol for the transfection of insect cells with virus DNA and transfer plasmid DNA (or bacmid DNA). 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 in **Subheading 3.2.** If the newer bacmid technologies are used, e.g., *flashBAC* 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 in **Subheading 3.4.** The virus amplification method described in **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.**) before proceeding to expression studies. **Sub-**

**heading 3.6.** contains a generic method for the transformation of Bac-to-Bac DH10Bac cells with pFastBac donor plasmids and **Subheading 3.7.** provides a method for the isolation and purification of recombinant bacmid DNA, ready for transfection of insect cells (**Subheading 3.1.**). More details on these specific protocols can be found on the Invitrogen website ([www.invitrogen.com](http://www.invitrogen.com)).

## 2. Materials

### 2.1. Cotransfection 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  $\mu$ L at 20 ng/ $\mu$ L) per cotransfection.
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  $\mu$ L at 100 ng/ $\mu$ L) per cotransfection (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, MD). Alternatively, any laboratory working with the baculovirus expression system may be able to help. Further details on insect cell culture can be found in **ref. 12** and in Chapters 6, 7, and 9.
4. 35-mm Tissue culture treated dishes seeded with insect cells (*see step 3*) in a subconfluent monolayer.
5. Serum-free insect cell culture medium. 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® (Invitrogen), FuGENE 6 (Roche), GeneJuice® (Novagen), 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 pipets, bijoux, or similar vessels to prepare the transfection. Plastic ware 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, as they produce distinct, large plaques in 3 d, compared to smaller less distinct plaques in 4 d for Sf-9 or -21 cells grown in serum-free medium.

3. Cotransfection medium to be titrated (from **Subheading 3.2.**).
4. Appropriate culture medium for the cells being used.
5. Low gelling temperature agarose for cell culture (use 2% w/v in sterile dH<sub>2</sub>O, 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 U/mL penicillin G sodium and 5  $\mu$ L/mL streptomycin sulfate 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- $\mu$ m 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 to distinguish *lacZ*-positive plaques from *lacZ*-negative plaques.
11. Sterile pipets, 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 pipets.
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 -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), magnetic stirrer, or other.
5. Incubator set at 27–28°C. (For spinner flask or tissue culture flask culture.)
6. Inverted phase-contrast microscope.
7. Sterile pipets.
8. Disinfectant for discard.

### **2.5. Titration of Virus by Plaque-Assay**

1. 35-mm Tissue culture treated dishes (10 dishes per virus to be titrated).
2. Insect cells (Sf-9 or -21 cells) taken from a healthy, exponential growth phase culture. The use of Sf-21 cells in serum-supplemented medium is strongly rec-

ommended for plaque-assays, as they produce distinct, large plaques in 3 d, compared to smaller less distinct plaques in 4 d for Sf-9 or -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<sub>2</sub>O, 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 U/mL penicillin G sodium and 5 µL/mL streptomycin sulfate in 0.85% saline; 1:100 final dilution). Antibiotic use is optional but if used should be added to all medium.
7. Incubator at 27–28°C and a plastic sandwich box.
8. 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 pipets, tips, and 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. 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 8.0).
2. *E. coli* DH10Bac cells ready for transformation (supplied by Invitrogen as part of a kit or separately).
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 Subheading 2.6., item 3.*).
5. 15-mL Disposable, sterile centrifuge tubes.
6. 42°C Water bath
7. 37°C Shaking and nonshaking incubator.

### **2.7. 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 sodium dodecyl sulfate. 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. 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE).

### 3. Methods

#### 3.1. *Cotransfection of Insect Cells With Virus DNA and Transfer Vector DNA*

1. For each cotransfection to make a recombinant virus, you will require one 35-mm dish of insect cells (Sf-9 or -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 subconfluent. As a guide, use  $1.0\text{--}1.5 \times 10^6$  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 cotransfection mix of DNA and liposome reagent. For each cotransfection, pipet 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  $\mu$ L 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 pipet; 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 cotransfection. 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 more. When removing liquid from a dish of cells, tip the dish at a 30–60° angle so the liquid pools toward 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 d in total, according to the recommendations given with the source of baculovirus vector DNA. For example, with the *flashBAC* system (Oxford Expression Technologies) 5 d 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 required (*see Notes 3–5*).
14. With the exception of the *flashBAC* 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 in **Subheading 3.2**. With the *flashBAC* 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 subconfluent monolayer (normally  $1.4 \times 10^6$  Sf-21 cells or  $0.9 \times 10^6$  Sf-9 cells/dish) (*see Note 6*). Leave the dishes for 1 h, on a level surface, at room temperature for the cells to recover.
2. During this incubation period prepare serial log (1 in 10) dilutions of the transfection medium. Prepare dilutions from  $10^{-1}$  to  $10^{-31}$ . 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 microcentrifuge tubes).
3. Add 50  $\mu$ L of undiluted transfection medium into the first bijou (this will be  $10^{-1}$ ) and mix thoroughly by vortex or inversion. Using a fresh pipet tip, remove 50  $\mu$ L 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 (in **step 1**), check that the cells have formed an even, subconfluent 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 is 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  $\mu$ L from each of the dilutions from  $10^{-1}$  to  $10^{-3}$  to triplicate dishes of cells and add 100  $\mu$ L undiluted transfection mixture to three other dishes. Add the diluted virus dropwise to the center of each dish, using a fresh sterile pipet for each, and label accordingly. Also include two dishes as controls where 100  $\mu$ L 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 1 × 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, ensure 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 pipet. 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 pipet 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.
11. Place the dishes into a secure container (e.g., a sandwich box) and incubate at 28°C for 3 d (Sf-21 cells) or 3–4 d (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 or X-gal to visualize the plaques. Plaques are clear areas against a red background as only live cells take up the Neutral Red stain. *LacZ*-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. *LacZ*-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), 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 the 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 pipet (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 -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 subconfluent 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 d) (*see* **Note 9**). In monolayer flasks (75 cm<sup>2</sup>), remove the medium and add approx 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<sup>2</sup> 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 mo or longer in the dark at 4°C. The titer of the virus will start to fall after a time and after storage for more than 3–4 mo it is recommended to titer the virus before using it—it may require reamplification. 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 reamplified and retitered when thawed. Do not store virus at –20°C or in liquid nitrogen.

4. Before using the virus in experiments, it is strongly recommended that it is titrated by plaque-assay to determine an accurate titer (**Subheadings 2.5. and 3.5.**) (*see Notes 10–14*).

### 3.5. Plaque Assay to Titer Amplified Virus

Alternative methods of titering the virus are given in Chapters 5, 10, 11, and 21.

1. To titrate an amplified virus by plaque assay, seed 10 dishes of cells per virus. Seed the dishes with an appropriate number of cells to form a subconfluent 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 microcentrifuge tubes).
3. Add 50  $\mu$ L 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 pipet tip, remove 50  $\mu$ L 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**), check that the cells have formed an even, subconfluent 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  $\mu$ L from each of the dilutions from  $10^{-7}$  to  $10^{-4}$  to duplicate dishes. Add the diluted virus dropwise to the center of each dish, using a fresh sterile pipet for each, and label accordingly. Also include two dishes as controls where 100  $\mu$ L 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 1 × 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. 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 pipet. 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 pipet 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 d (Sf-21 cells) or 4 d (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 or X-gal to visualize the plaques. Plaques are clear areas against a red background as only live cells take up the Neutral Red stain. *LacZ*-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. (*LacZ*-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. 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  $\mu$ L thawed cells into a 15-mL centrifuge tube (prechilled on ice). Then add 1 ng (5  $\mu$ L) 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  $\mu$ L LB medium (or similar) and shake cells at 37°C for 4 h to allow expression of the antibiotic resistance markers.
7. Prepare 10-fold serial dilutions of the cells ( $10^{-1}$  to  $10^{-31}$ ) with LB and plate out 100- $\mu$ L aliquots of each dilution onto selective agar plates (**Subheading 2.6.**). 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 gray 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 restreaking onto selective agar plate (**Subheading 2.6.**) 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 3.7.**).

### **3.7. 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 3.6.**) 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 14,000g 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 14,000g 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 14,000g 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  $\mu$ L sterile TE. Store at 4°C (not frozen). Do not shear the DNA by pipetting too often. Keep sterile because 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 analyzed 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 ([12](#)).
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 previously listed instructions have been followed and the insect cells are in good condition, then the recombinant virus titer produced after cotransfection will normally be high (extensive testing in our labs indicate an average titer of about  $10^7$  pfu/mL at 5 d).
4. The cells remaining from the cotransfection 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 cotransfection is not adversely affected by using semi-pure transfer plasmid DNA (e.g., that produced by resin-based miniprep DNA protocols); however, the foreign gene expression levels in these initial infected cells is 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 ( $\gg 1$  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 to have a synchronous culture, then it is critical that the cells be infected with a known and relatively high MOI (a 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 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  $\times$  dilution factor\*  $\times 10^{**}$   
 \*multiply by the inverse of the dilution used on the plate used to count the plaques and \*\*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 all the cells in culture (*see* **Note 7**).

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

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### Summary

BacMams are modified baculoviruses that contain mammalian expression cassettes for gene delivery and expression in mammalian cells. The BacMam system combines the advantages of viral transient expression, ease in generation, and a wide cell tropism. It enables rapid, facile, and flexible gene over-expression experiments to be performed in a variety of mammalian cell lines. Conversion of baculovirus vectors to BacMam vectors involves replacement of the viral specific expression cassette with a mammalian expression cassette or the addition of a mammalian expression cassette. Viruses are produced using standard methods in a few weeks. Mammalian cells transduced with the BacMam viruses have been routinely used as substitutes for stable cell lines.

**Key Words:** BacMam; modified baculovirus; transient expression; viral transduction; gene over-expression; coexpression; GPCR; FACS.

### 1. Introduction

With the convergence of systems biology and availability of results from functional genomic and proteomic studies, researchers are provided a better picture on the functional interplay of many target molecules. As a result, multiple genes or multiple variants of a single gene are often simultaneously studied. These tasks require an efficient, facile, and transient system to deliver genes to a variety of cultured mammalian cells to allow expression and coexpression studies. The BacMam technology provides a powerful tool for these applications. Since the initial demonstration that baculovirus can efficiently transduce mammalian cells (1,2), it has gradually gained its popularity as a tool for gene delivery to cultured mammalian cells (3–5). The term BacMam was coined to specifically refer to baculoviruses in which a mamma-

lian promoter is used to drive gene expression in mammalian cells following viral transduction. With the use of a number of improved methods, recombinant BacMams and baculoviruses can be easily generated using viral genomes constructed in *Escherichia coli* cells (6). To avoid confusion, we will use the term “baculovirus” for viruses made for gene expression in insect cells, and “BacMam” for those modified to contain a mammalian expression cassette for gene expression in mammalian hosts.

BacMams derived from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV [6]) are able to transduce a variety of mammalian cell lines, including HEK293, HeLa, U-2 OS, monkey kidney (COS), and Chinese Hamster ovary (CHO) cells (3,5). The viral genome can stably accommodate an insert sequence of at least 38 kb (8), making expression of large and multiple genes possible. Unless a selection force is applied, gene expression in transduced cells is transient and can usually last for up to 4 d. However, with a proper viral transduction and feeding procedure, expression can even be prolonged to 16 d (9). For viruses carrying a selectable marker, stable cell lines can also be established upon selection (10,11). Compared to other human-derived viral delivery vectors, the safety requirements for handling baculoviruses and BacMams are relatively low (12,13). Given the nature and origin of the virus, this commonly used form of recombinant baculovirus is unable to replicate in mammalian cells, can be manipulated in laboratories at BSL1/2 levels and can be easily inactivated by treating with 70% ethanol (12,13). Insect larvae in the wild are infected via the gut by occluded baculoviruses; the genetically engineered polyhedrin-deleted virus does not efficiently infect larvae. The viruses are unstable outside of the laboratory, so they are environmentally safe as well (14).

BacMams have been used as a delivery vehicle to mammalian cells for many gene classes, including nuclear receptors (15), secreted proteins (16,17), and membrane proteins, e.g., G protein-coupled receptors (GPCRs) (18–20) and ion channels (21). Applications range from functional characterization and protein production, to cell membrane generation and assay development. With its high transduction efficiency and flexibility, the technology easily enables coexpression applications and modulation of expression level by dosing and timing. This type of flexibility is especially important in gene function studies and assay development processes where mix-and-match coexpression experiments with a number of cofactors and interacting partners are often necessary (Fig. 1). BacMam stocks, supplemented with 5% heat-inactivated fetal bovine serum (FBS) as described in the protocol, are stable when stored at 4°C in the dark. Mammalian cell assays can be very sensitive, so it is important to be aware of responses that could arise from FBS, media components, or proteins released into the insect medium during viral amplification.

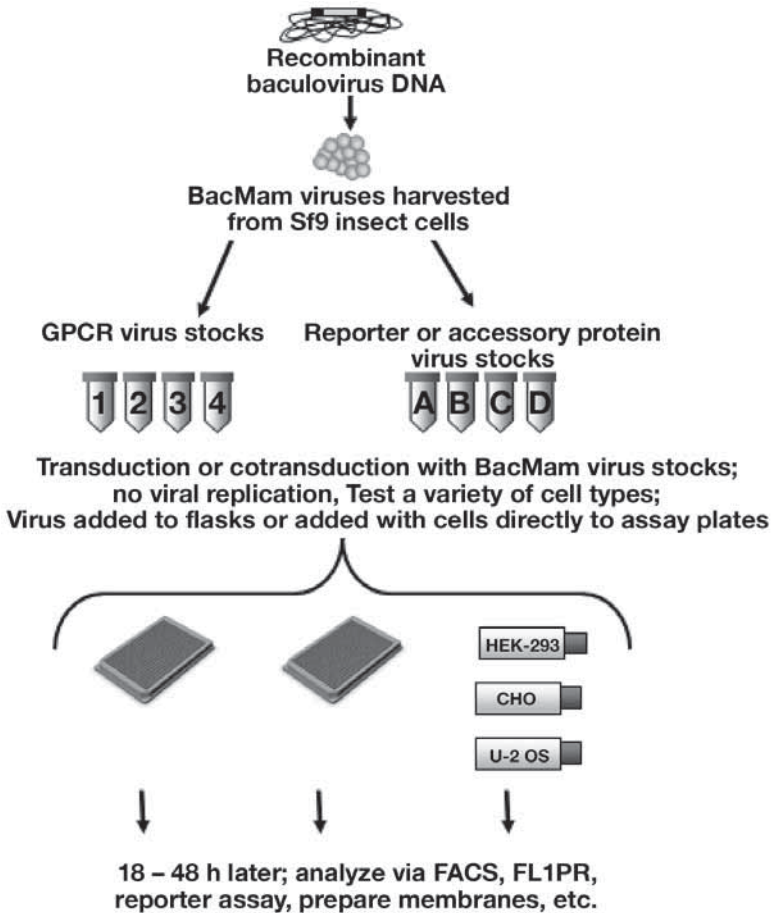


Fig. 1. Schematic representation of BacMam generation and subsequent transduction of mammalian cells for functional studies and assay development.

These BacMam stocks provide the means to quickly perform newly designed experiments with a few routinely used mammalian host cell lines. In contrast, these tasks are more difficult to achieve in a timely fashion by the stable cell line approach. The BacMam technology and its applications are discussed in more depth in a few recent reviews (3–5,19,22). We have generated BacMams for more than 200 GPCRs and their cofactors and have used them to develop functional cell based assays for many of them. We describe here construction of a few BacMam transfer vectors and protocols routinely used in our laboratories for BacMam generation, together with a few selected applications.

## 2. Materials

1. Baculovirus transfer vector pFastbac1 (Invitrogen, Carlsbad, CA).
2. Mammalian expression vector pcDNA3.1 (Invitrogen).
3. Restriction enzymes (*Sna*BI, *Stu*I, *Not*I, *Hpa*I, *Pvu*II, *Asc*I), T4 DNA ligase, agarose gels.
4. Plasmid purification kit (Qiagen, Waltham, MA).
5. Sf-9 cells (Invitrogen).
6. Insect cell serum-free medium EX-CELL 420 (JRH Biosciences, Lenexa, KS).
7. Heat-inactivated fetal bovine serum (JRH Biosciences).
8. Luria Broth (LB) ampicillin plus gentamicin plates (with 50  $\mu$ g/mL ampicillin and 5  $\mu$ g/mL gentamicin).
9. *E. coli* strain DH10Bac (Invitrogen).
10. Kanamycin, tetracycline, IPTG.
11. Gentamicin and Bluo-gal (Invitrogen).
12. DH10Bac plates: Luria Agar plates plus, 50  $\mu$ g/mL kanamycin, 3.5  $\mu$ g/mL gentamicin, 10  $\mu$ g/mL tetracycline, 40  $\mu$ g/mL IPTG, 100  $\mu$ g/mL Bluo-gal.
13. DH10Bac growth medium: LB medium supplemented with 50  $\mu$ g/mL kanamycin, 5  $\mu$ g/mL gentamicin, 5  $\mu$ g/mL tetracycline.
14. 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).
15. Transfection reagent Cellfectin (Invitrogen).
16. Centrifuge for cell culture (Beckman, Allegra 6, GH-3.8A rotor).
17. 500-mL and 3-L Shake flasks for Sf-9 cell cultures (Corning, Acton, MA).
18. Cell culture flasks T-25, T-75, and T-150 (Corning).
19. Sterile Falcon polypropylene culture tubes, 5, 15, and 50 mL (Becton Dickinson, Franklin Lakes, NJ).
20. The BacPAK Baculovirus Rapid Titer Kit (BD Biosciences).
21. HEK293 (ATCC, cat. no. CRL-1573).
22. DMEM/F12 medium for mammalian cell cultures (JRH).
23. Trypsin (JRH).
24. Versene (Invitrogen).
25. Dulbecco's phosphate buffered saline (DPBS).
26. Bovine serum albumin (Roche, Indianapolis, IN).
27. Buffer A (stored at 4°C) is DPBS supplemented with 0.5 mM MgCl<sub>2</sub>, add 2% BSA, and is sterile-filtered through a 0.2- $\mu$ M cellulose acetate filtration unit.
28. Anti-HA monoclonal antibody (Covance, Princeton, NJ).
29. Rabbit anti-mouse antibody, Alexa488 (Molecular Probes, Eugene, OR).

## 3. Methods

### 3.1. The BacMam Virus Generation System

The BacMam system described in this chapter is based on the Bac-to-Bac system (Invitrogen) for baculovirus generation. With this system the recombi-

nant baculoviral genome is constructed in *E. coli*, via a transfer vector. The gene of interest is first subcloned into a BacMam transfer vector, which is then transformed into a special *E. coli* strain DH10Bac (see **Subheading 3.5.1.**) to generate the recombinant viral DNA. The viral DNA is then 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 the procedure, recombinant BacMams can be generated in less than 2 wk.

The Bac-to-Bac system is based on the method of Luckow et al. (6), which uses the Tn7-mediated site-specific transposition reaction to direct integration of expression cassettes contained in the transfer vector into a baculovirus backbone vector (bacmid) preexisted in the *E. coli* strain DH10Bac. 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 resistant 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 in the *lacZ* $\alpha$  gene contained within the recombinant viral genome, causing inactivation of the  $\alpha$ -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.

### 3.2. BacMam Transfer Vectors

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 baculovirus. Described below are a few BacMam transfer vectors routinely used in our laboratories, together with a baculovirus transfer vector pFastBac1 that can be directly used as a BacMam transfer vector. We have not used the commercially available BacMam transfer vector pTriEx from Novagen.

#### 3.2.1. 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 has been directly 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). The mammalian expres-

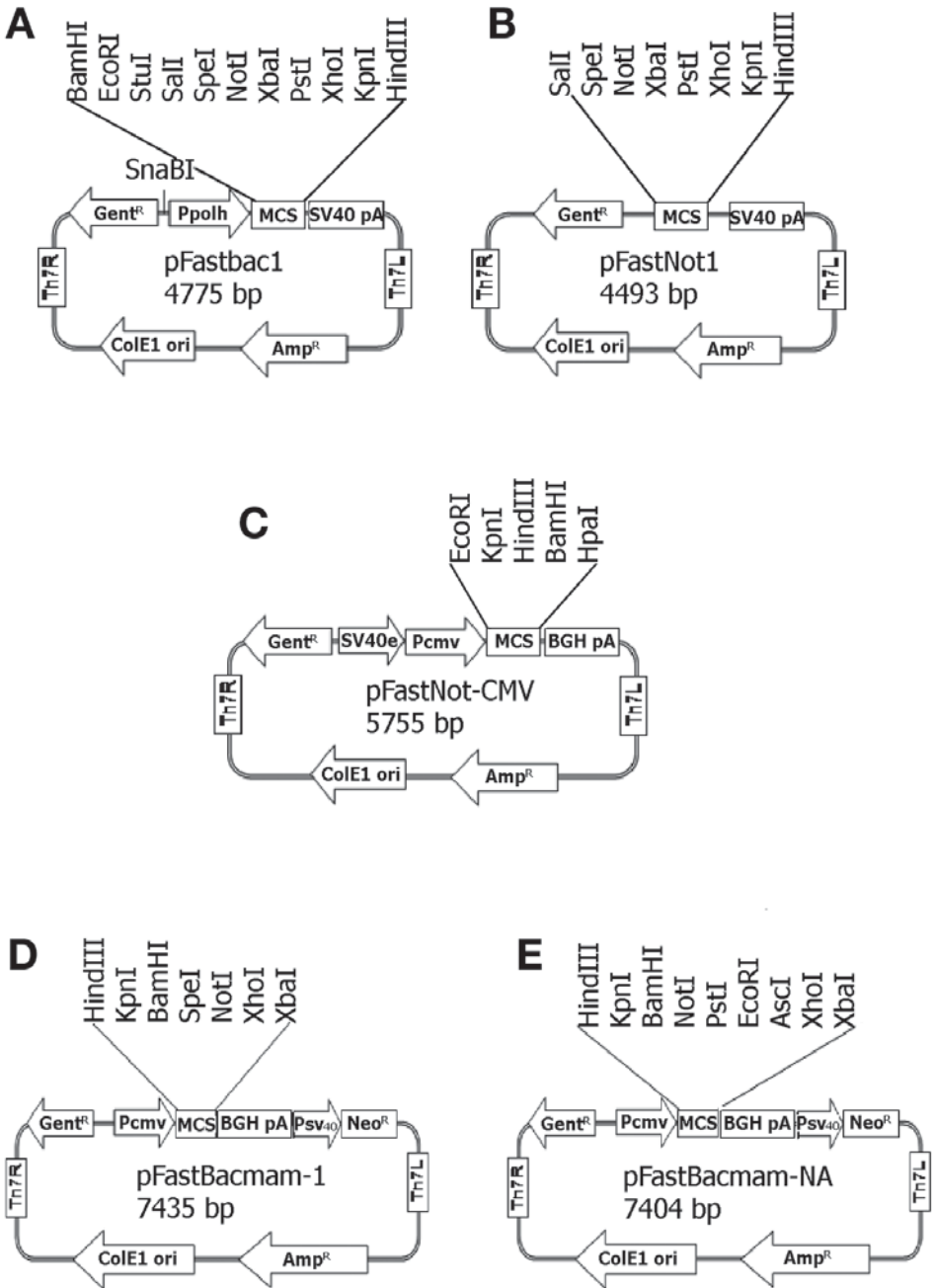


Fig. 2. BacMam transfer vectors.

sion cassette can also be used to replace the polyhedrin promoter using the unique *Sna*BI site in combination with another site in the MCS.

### 3.2.2. *pFastNot1*

*pFastNot1* (**Fig. 2B**) is a derivative of *pFastBac1* in that the polyhedrin promoter in *pFastBac1* is removed. This was done by deleting DNA sequences contained between *Sna*BI and *Stu*I sites in *pFastBac1*. The vector is smaller than *pFastBac1* in size, and is used to accommodate any DNA fragments with mammalian expression cassettes. *pFastNot1* is constructed as follows:

1. Digest 1 µg of *pFastBac1* with *Sna*BI and *Stu*I.
2. Self-ligate plasmid DNA (both restriction sites generate blunt ends).
3. Transform *E. coli* and identify plasmids deleted for the *Sna*BI-*Stu*I fragment.

### 3.2.3. *pFastNot-CMV*

*pFastNot-CMV* (**Fig. 2C**) is a derivative of *pFastNot1* in that sequences containing part of the MCS and the SV40 poly A in *pFastNot1* are replaced by a mammalian expression cassette containing a CMV promoter with a SV40 enhancer, a MCS, and the BGH polyadenylation sequence. This is achieved by replacing sequences between the *Not*I/*Hpa*I sites in *pFastNot1* with the *Not*I/*Pvu*II fragment derived from the mammalian expression vector *pCDN* (**23**). *pFastNot-CMV* is considered a minimal size BacMam vector that can accommodate any cDNA open reading frames (ORFs) for expression in mammalian cells. *pFNcmv* is constructed as follows:

1. Digest *pFastNot1* with *Not*I and *Hpa*I; treat with phosphatase.
2. Digest *pCDN* with *Not*I and *Pvu*II.
3. Ligate, transform *E. coli*, and select for both ampicillin and gentamicin resistance.
4. Identify plasmids with the inserted mammalian expression cassette.

### 3.2.4. *pFastBacMam-1*

*pFastBacMam-1* (**Fig. 2D**) has been described previously (**10**). The vector is a hybrid of *pFastBac1* and *pCDNA3* (Invitrogen), which contains the mammalian expression cassette with the CMV promoter, a MCS, and the BGH polyadenylation sequence. In addition, it also contains a neomycin resistance gene driven by the SV40 promoter. The neomycin resistance marker allowed selection of stable cell lines following BacMam transduction (**10,11**). *pFastBacMam-1* is constructed as follows:

1. Digest *pFastBac1* with *Sna*BI and *Hpa*I; treat with phosphatase.
2. Digest *pCDNA3* with *Nru*I and *Bst*1107I.
3. Ligate, transform *E. coli*, and select for both ampicillin and gentamicin resistance.
4. Identify plasmids with the inserted mammalian expression cassette.

### 3.2.5. pFastBacmam-NA

pFastBacmam-NA (**Fig. 2E**) is a modification of pFastBacMam-1. The vector was made by replacing part of its MCS contained between *Bam*HI/*Xho*I in pFastBacMam-1 with a synthetic sequence containing additional unique sites, including a *Not*I site (5'GCGGCCGC3') and an *Asc*I site (5'GGCGCGCC3'). pFastBacmam-NA can be used to accommodate cDNA ORFs as *Not*I/*Asc*I fragments from Gateway™ vectors (Invitrogen) such as pENTR/D-TOPO, pENTR/SD/D-TOPO, pcDNA3.2/GW/D-TOPO, pcDNA6.2/GW/D-TOPO, and any other derivative (destination) vectors that acquired gene ORFs from the previously listed vectors via Gateway recombination reactions.

### 3.3. cDNA

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 will be subcloned from a common vector it is useful to configure a BacMam transfer vector with compatible restriction sites.

### 3.4. Subcloning Into the Transfer Vector

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 an appropriately digested transfer vector DNA.
3. Transform *E. coli* (TOP10, DH5 $\alpha$ , and so on) 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.

### 3.5. Generation of Bacmid DNA

#### 3.5.1. Transformation of DH10Bac

1. Transform 15–20  $\mu$ L of DH10Bac competent cells (Invitrogen) with 10 ng transfer plasmid.
2. Incubate on ice for 30 min.
3. Heat shock 42°C for 30 s.
4. Add 1 mL recovery broth to a 15-mL culture tube; add cells.
5. Incubate at 37°C for 3–4 h.
6. Plate different amounts of culture on four DH10Bac plates (e.g., 10, 50, 90, 150  $\mu$ L).
7. Incubate at 32°C for 2 d.
8. Pick three large white colonies; streak each for single colonies on a DH10Bac plate (*see Note 2*).
9. Incubate at 32°C for 2 d.

### 3.5.2. Preparation of Bacmid DNA

Bacmid DNA is isolated using a modified protocol of the alkaline lysis method (24). The buffers included in the Qiagen DNA Maxi Preparation Kit are used, but solutions prepared from laboratory chemical stocks work as well.

1. Examine the plates of restreaked colonies generated under **Subheading 3.5.1**.
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 at 34°C (see **Note 3**).
5. Transfer 1.4 mL of the overnight culture to a 1.5-mL microfuge tube; pellet the cells.
6. Resuspend the pellet in 200 µL P1; add 200 µL P2; invert to mix.
7. Add 200 µL P3; spin in a microcentrifuge for 10 min to pellet debris.
8. Transfer supernatant to a sterile 1.5-mL microfuge tube. Add 420 µL isopropanol and store at -20°C.
9. Spin in a microcentrifuge to pellet DNA. Dry DNA and resuspend in 50 µL dH<sub>2</sub>O.

### 3.6. Generation of BacMam Virus

All tissue culture work and the viral work are performed in a biosafety cabinet to maintain the sterility of the BacMam stocks for subsequent applications.

#### 3.6.1. Sf-9 Cell Culture

Sf-9 cells are excellent hosts for producing BacMam virus. Sf-9 cells double in 18–24 h, and grow very well as single cells in suspension (see **Note 4**) in EX-CELL 420 serum free medium (JRH Biosciences). Cells adapted to EX-CELL 420 have performed well for many passages (see **Note 5**), and suspension cells are easily scaled to large volumes as needed (e.g., see Chapter 11).

#### 3.6.2. Transfection of Sf-9 cells

1. Exponentially growing cells (see Chapter 1) are desired for transfection. Approximately  $3 \times 10^6$  cells in 3 mL of EX-CELL 420 are seeded into a T-25 cell culture flask (see **Note 6**). The cells are allowed to attach for 30 min.
2. For each transfection, add 25 µL of bacmid DNA (from **Subheading 3.5.2**) 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 EX-CELL 420 medium for each sample.
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 mL of EX-CELL 420 medium supplemented with 10% heat inactivated FBS to the flask.
8. Cover the flasks with a sheet of aluminum foil and continue the incubation for another 4 d at 27°C.

### 3.6.3. Harvesting the P0 Virus

Cytopathic effect is usually evident 3–4 d after bacmid transfection. The areas of clearing (inhibition of cell growth and lysis of cells) should be visible when the T-flask is held to a light.

1. Transfer the conditioned culture medium from the T-flask to a conical 15-mL culture tube.
2. Centrifuge to pellet the cell debris 10 min at 650g (Beckman GH3.8 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 and are typically around  $0.5\text{--}1.0 \times 10^7$  plaque forming units per milliliter (pfu/mL).

### 3.6.4. Generating P1 Virus

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

1. Seed culture to  $7.0 \times 10^5$  cells/mL; incubate 27°C overnight on a shaker at 120 rpm.
2. Cultures typically grow to  $1.5\text{--}2.0 \times 10^6$  cells/mL, count samples from a few flasks.
3. Add heat-inactivated FBS (JRH Biosciences) to a final concentration of 5% (*see Note 8*).
4. Infect with 1 mL P0 and incubate on a shaker (120 rpm) at 27°C for 4 d in the dark.
5. Count the cells the day after infection to confirm that they have not doubled (*see Note 9*).
6. Transfer the culture to a 250-mL conical centrifuge bottle; pellet debris at 3600g at 4°C for 20 min.
7. The medium supernatant (P1 stock) is stored at 4°C in the dark.

The culture is transferred to a 250-mL centrifuge bottle and clarified by centrifugation at 3600g at 4°C for 20 min. The medium supernatant (typically referred to as a P1 stock) is stored at 4°C in a sterile Nalgene bottle in the dark. This P1 BacMam stock typically reaches a titer of  $10^8$  pfu/mL, and following clarification by low speed centrifugation can be used to transduce mammalian cells.

### 3.6.5. Large-Scale Amplification of BacMams

For larger volumes of virus stocks, 1 L virus stocks can be generated. This P2 BacMam stock typically reaches a titer of  $10^8$  pfu/mL.

1. Transfer 1 L of EX-CELL 420 medium to a 3-L Corning shake flask.
2. Seed Sf-9 cells to  $7.0 \times 10^5$  cells/mL, incubate culture on a shaker (55 rpm) at 27°C in dark.
3. After 24 h or when the cell counts reach  $1.5\text{--}2.0 \times 10^6$  cells/mL, 1 mL P1 stock is added (*see Note 10*).
4. Add heat-inactivated FBS to 5% (*see Note 8*).
5. After 4 d, transfer the culture to 500-mL centrifuge bottles.
6. Pellet cell debris at 3600g for 20 min.
7. Decant virus into sterile bottles and store at 4°C in the dark.

### 3.7. BacMam Titration

Virus titration can be time consuming, but should be part of the standard quality control process. Any standard method for viral titration can be used. The BacPAK Baculovirus Rapid Titer Kit (BD Biosciences) uses an anti-gp64 antibody to detect viral plaques on Sf-9 cell lawn, and it takes 2 d to perform. The kit includes a complete protocol manual. A brief outline of the protocol is included here. (Note that alternative methods of titering the virus are given in Chapters 4, 10, 11, and 21.)

1. Seed a 96-well plate with Sf-9 cells.
2. Infect plate with dilutions of virus.
3. Incubate 1 h; remove virus inoculum; overlay with methyl cellulose.
4. Incubate 2 d 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.

### 3.8. Long Term Storage of BacMams

BacMam virus stocks (P1 or P2) are stable for longer than 1 yr when kept in the dark at 4°C (*see Note 8*). However, titers of P0 stocks will drop over time but can be used for many years for the generation of P1 stocks.

### 3.9. Virus Purification

Procedures for recombinant baculovirus purification have been described (25). However, for BacMam transduction into cultured cells, it is not necessary to purify the virus, i.e., the P1 and P2 stocks can be directly used (*see Note 11*).

### 3.10. Gene Delivery to Mammalian Cells by BacMam Transduction

In almost all the cases, BacMam transduction of mammalian cells is performed in the same medium and incubated at the same temperature (37°C, 5% CO<sub>2</sub>) that is used to propagate the mammalian host cells (*see Note 12*).

#### 3.10.1. Identification of Receptive Mammalian Cell Lines

BacMam has been shown to be able to transduce a variety of mammalian cell lines (*see Tables in refs. 3 and 10*). However, transduction efficiency varies among different cell lines. Discrepancies in transduction efficiency are even observed among isogenic cell lines derived from different lineage or sources. For example, a freshly isolated clonal CHOK1 cell line appears to be more susceptible to BacMam transduction compared to a “standard” laboratory CHOK1 line, and one HEK293 cell line may perform better than another line from a different lineage (unpublished observations). Nonetheless, the BacMam transduction efficiency of any given cell line can be determined by one of the following methods. One method is to use a BacMam expressing green fluorescent protein to transduce the cell line at various MOI and examine the percent of green fluorescent cells after 24 h. Another way is to use an antibody to detect the recombinant protein after BacMam transduction. A common approach for immunodetection of GPCRs is the addition of an amino-terminal HA-tag (*see Note 13*). Examples of both assays are presented next.

#### 3.10.2. Transduction of Cells With BacMam Virus

##### 3.10.2.1. TRANSDUCTION OF ADHERENT MAMMALIAN CELLS

Adherent cells (e.g., HEK293, CHOK1, U-2 OS) are maintained in T-150 flasks in DMEM + 10% FBS (complete medium) and passed every 4–5 d 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) suspension cultures are often preferred.

1. Aspirate the growth medium. Wash the cell layer with 10 mL 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. Centrifuge at 162g for 10 min, and resuspend the cells in 10 mL complete medium.
2. Count the cells and plate  $1.5 \times 10^6$  cells in 15 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 a titer of approx  $10^8$  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  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .
5. Aspirate the medium, wash with 5 mL PBS/0.1 mM EDTA (*see Note 15*). Dislodge cells and transfer cells to 15 mL complete medium in a 50-mL conical tube. Centrifuge at 162g, and resuspend the cells in 10 mL complete medium.

#### 3.10.2.2. 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 (*17*). Described next is a procedure that we use to transduce a CHOK1 suspension culture with BacMam. CHO K1 suspension cells grow well in EX-CELL 302 medium (JRH Biosciences) and are usually split every 3–4 d.

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  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  with shaking (120 rpm).
2. Add sufficient virus stock to obtain an MOI of approx 15 and sodium butyrate to 5 mM, continue shaking for 24–48 h.
3. Harvest cells and process or assay by any established methods.

#### 3.10.2.3. STAINING CELLS FOR FLUORESCENCE-ACTIVATED CELL SORTING ANALYSIS

The following protocol is used for fluorescence-activated cell sorting (FACS) analysis of an intracellular expressed protein. For this purpose, cells are fixed and permeabilized prior to antibody staining. For detecting extracellular domains of membrane proteins, aliquot  $5 \times 10^5$  cells into 2 mL Buffer A, pellet the cells, and proceed from **step 8**.

1. Prepare a T-75 of transduced cells for each BacMam virus, harvested with Versene and resuspended in complete cell culture medium, as outlined in **Sub-heading 3.10.2.1. or 3.10.2.2.**
2. Transfer  $10^6$  cells to a 50-mL centrifuge tube. After adding 5 mL PBS, the cells are pelleted by centrifugation at 162g for 10 min.
3. Aspirate the supernatant; resuspend the cells in 1.4 mL PBS and add 0.2 mL diluted formaldehyde solution (0.54 mL of 37% stock in 9.5 mL PBS).
4. Incubate on ice for 30 min, and add 4 mL PBS. The cells are then pelleted by centrifugation at 162g for 10 min.
5. Aspirate the supernatant, add 2 mL 0.2% Tween/PBS to the cells.
6. Incubate 15 min  $37^\circ\text{C}$ ; add 4 mL PBS; transfer 3-mL aliquots to Falcon 2054 tubes. Pellet the cells at 80g for 5 min.
7. Prepare primary antibodies for staining as follows. Dilute anti-HA at 1:250 in Buffer A (use 0.25 mL for each cell sample). If appropriate, also prepare a negative control isotype (e.g., anti KLH) at 1:250.

8. Aspirate buffer from cells, resuspend gently, and add 0.25 mL diluted antibody. Place in ice on a shaker (80 rpm) for 30 min.
9. Add 2 mL Buffer A to wash cells after primary stain, centrifuge, aspirate. Repeat the wash once.
10. Prepare secondary antibody as follows. Dilute labeled secondary antibody (e.g., anti-mouse Alexa 488 as previously listed, 1:250 [use 0.25 mL for each sample]). Aspirate Buffer A from cells, resuspend, and add 0.25 mL secondary antibody. Cover the tube with a cap or foil, and place the tube in ice on a shaker (80 rpm) for 30 min.
11. Wash as in **step 9**.
12. Resuspend cells in 0.5 mL Buffer A. The cells are ready for FACS analysis or they can be stored at 4°C in dark.

### 3.10.3. Examples of Gene Expression Studies in BacMam Transduced Cells

Expression of genes in mammalian cells by BacMam transduction can be analyzed by established methods, such as Western blot, TaqMan, FACS, and others. This section shows a few examples in using the technology. At a single cell level, FACS analysis provides useful data on the percentage of cells expressing the target gene and the relative level of detectable expression within each cell. Cells transduced with a BacMam expressing green fluorescent protein and harvested as previously described (**Subheading 3.10.2.**) can be analyzed without further manipulation. **Figure 3** shows such an experiment to demonstrate how expression levels can be modulated. In the experiment, different MOIs were used. At the lowest MOI, a significant proportion of the cells fluoresce at very low levels (peak on the left). With increasing MOI, the fluorescent signal from transduced cells increases significantly (**Fig. 3**).

FACS analysis can also be used to study transduced cells using antibodies labeled with a fluorescent dye. An example is shown in **Fig. 4**. HEK293 cells were transduced with a BacMam expressing human GPR7, with an engineered HA epitope at its amino terminus, using the protocol described in **Subheading 3.10.2.1**. The peak on the left side of the histogram was derived from mock-stained cells to indicate the nonspecific background. The peak on the right side of the histogram represents the cells within the transduced population that have the HA-tagged receptor displayed on the surface. The relatively narrow peak indicates the uniform receptor expression levels within the transduced population. There are also relatively few low fluorescence (untransduced) cells (**Fig. 4**).

A functional assay can also be used to analyze BacMam transduced cells. **Figure 5** shows an experiment using a BacMam to express a GPCR, the human dopamine D1 receptor, in a HEK293-Luc reporter strain (**27**). The experiment

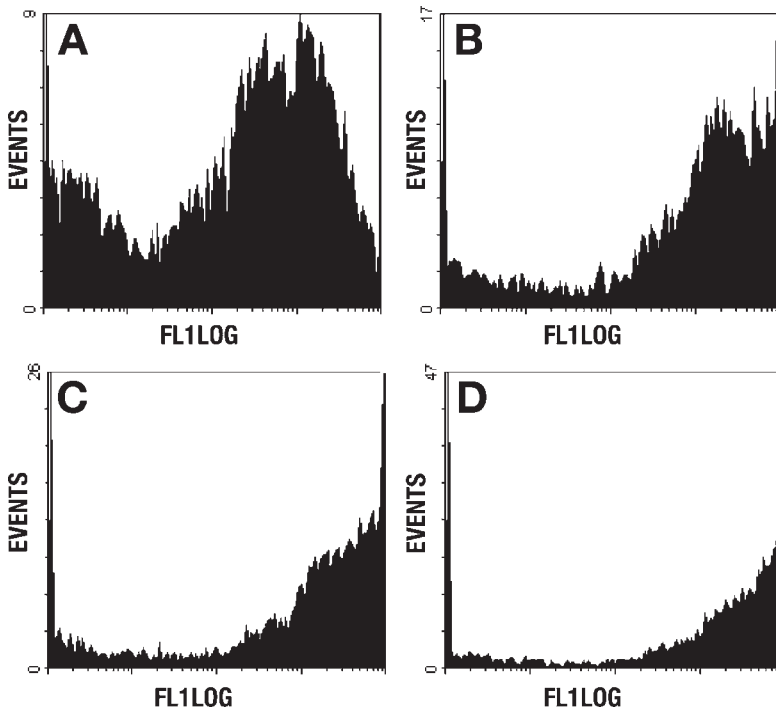


Fig. 3. Fluorescence-activated cell sorting analysis of HEK293 cells transduced with BacMam expressing green fluorescent protein (GFP). HEK293 cells were transduced in a T-75 flask with varying amounts of BacMam virus expressing GFP. A, 0.05 mL (MOI ~3); B, 0.25 mL (MOI ~15); C, 0.5 mL (MOI ~30); D, 1 mL (MOI ~60).

was performed using a modified protocol of that described in **Subheading 3.10.2.1**. The HEK293-Luc strain contains a stably integrated CRE-Luc reporter for detection of intracellular cAMP changes upon ligand–receptor interactions. It is clear that in HEK293-Luc cells expressing BacMam-delivered receptor significant levels of luciferase activity were detected upon dopamine treatment, whereas only background levels of luciferase activity were detected in nontransduced cells (**Fig. 5**).

#### 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 pFastBac vectors can be

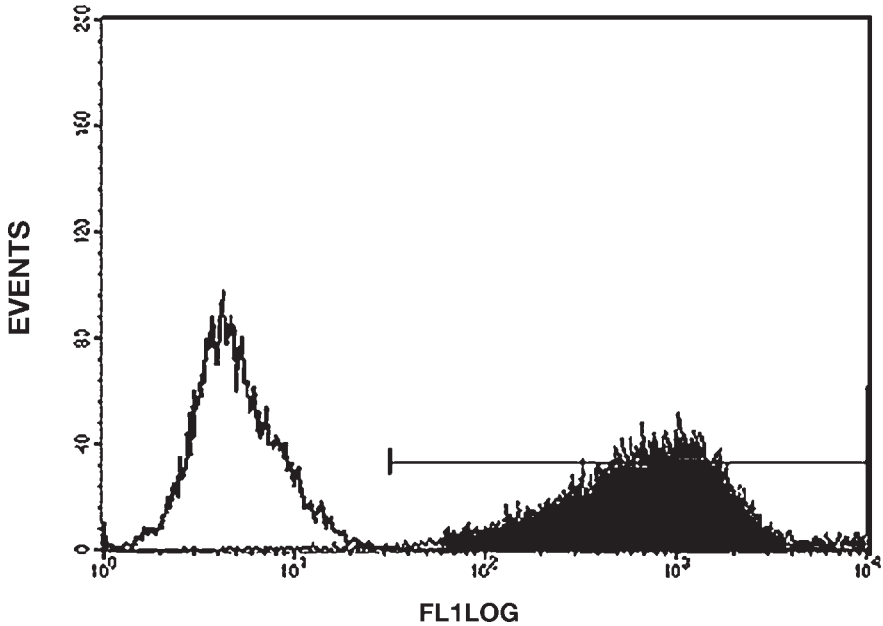


Fig. 4. Fluorescence-activated cell sorting analysis (FACS) analysis of HEK293 cells transduced with a BacMam expressing HA-GPR7. FACSscan with Flowlink software was used. Cells were transduced and stained as described in the protocol.

simplified by using a “shotgun” approach. This can be done by directly ligating a restriction-digested donor plasmid DNA with a properly prepared pFastBac 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.

2. The DH10Bac transformants can also be incubated at 37°C for >24 h. 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 to be lysed.
4. Sf-9 cells adapt easily to suspension. A vial of frozen cells is recovered in a T-flask containing serum-free medium. After 3 d, the medium is removed and the attached cells are scraped into fresh serum-free medium, maintaining a minimum of 10<sup>6</sup> viable cells/mL. After a few days, the cells are split to 10<sup>6</sup> cells/mL and thereafter split 1:10 every 3–4 d.

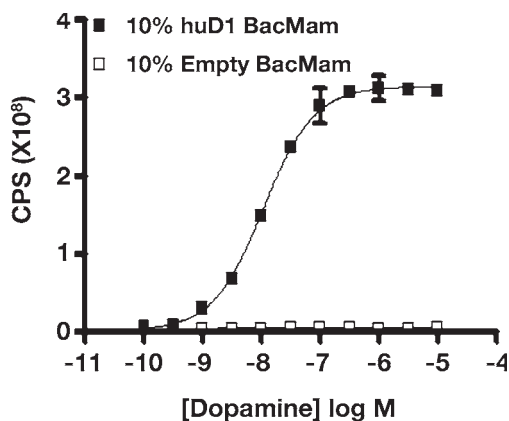


Fig. 5. Luciferase reporter assay of HEK293-Luc cells transduced with a BacMam expressing the human dopamine D1 receptor. HEK293-Luc cells were seeded into a T-75 flask at  $4.5 \times 10^6$  cells/flask. Following overnight incubation at  $37^\circ\text{C}$ , BacMam virus expressing the human dopamine D1 receptor was added to the flask. The cells were then incubated at  $37^\circ\text{C}$  for 2 d, harvested, resuspended, and added into Matrigel-coated 96-well plate at 30,000 cells/well. The plate was incubated at  $37^\circ\text{C}$  overnight. Following the addition of isobutylmethylxanthine to  $30 \mu\text{M}$  and several different concentrations of dopamine, the plate was incubated at  $37^\circ\text{C}$  for 6 h. The luciferase reporter assay was performed using the Luclite kit and a TopCount-HTS (both from Perkin Elmer).

5. We have found that Sf-9 cells cultured in EX-CELL 420 will maintain a homogeneous culture for more than 100 passages. With some other commercial preparations the cell population changes with passage, 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 commonly added the next day.
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. 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. One milliliter of P0 is usually added to 150 mL of cells, which is sufficient to cause growth cessation within the first 24 h. Infected cells are usually larger, with irregular shapes. Cell viabilities at harvest vary over a wide range. For some constructs significant cell lysis also 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 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, the cell numbers during amplification can rise above the desired limit for the medium and cell line in use ( $2.0\text{--}4.0 \times 10^6$  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. There are several approaches to resolve the problem. The virus can be concentrated and resuspended in PBS or 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.
12. High level expression of a GPCR is usually not required for many of the functional assays we perform. Numerous examples of transducible mammalian cell types have been reported (3,5,10,22). An investigation of optimal conditions for BacMam transduction of mammalian cells has been published (26).
13. The amino termini of GPCRs vary in length and are usually extracellular. The influenza hemagglutinin epitope (YPYDVPDYA) is commonly added to the N-terminus of GPCRs following the signal sequence. Cells expressing the tagged GPCR can be stained with anti-hemagglutinin antibodies to determine if the receptor is localized on the cell surface.
14. We and others have used cells transduced with BacMams as replacements for stable cell lines for various applications (15–21).
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, trypsin can safely be substituted to improve the yield of cells.

## Acknowledgments

The authors thank Tom Kost and J. Patrick Condreay for discussion and advice. We also wish to thank Gordon McIntyre and Mike Romanos for their support of BacMam technology development and applications.

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## Available Lepidopteran Insect Cell Lines

Dwight E. Lynn

### Summary

This chapter lists the known cell lines from Lepidoptera, largely based on previous compilations of insect cell lines published by W. Fred Hink. 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; insect cells; virus susceptibility; established cell lines; tissue source.

### 1. Introduction

Early in the history of insect cell culturing, researchers in the field began meeting at 3- to 4-yr 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). Unfortunately, the last of his compilations was published 15 yr ago, so a literature search has been performed for publications since that time. As seen in Fig. 1, the availability of lepidopteran cell lines has steadily increased at about 50 new lines per decade. In addition to details on the insect species, designation of the resulting cell lines, and tissue source used, details on the susceptibility to baculoviruses have also been provided where they are known. Finally, the baculoviruses that have been grown in insect cell culture are summarized in Table 2. Note that a given baculovirus is named based on the insect species from which it was first isolated.

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

**Table 1**  
**Lepidopteran Cell Lines E**

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
<i>Adoxophyes orana</i>	Ao/I	Adult ovaries	AcMNPV, AdorMNPV, MbMNPV, SfMNPV	NP-1
<i>Adoxophyes orana</i>	FTRS-AoL1	Neonate larvae	AdorMNPV, PlxyMNPV	<a href="#">8</a>
<i>fasciata</i>	FTRS-AoL2	Neonate larvae	AdorMNPV, PlxyMNPV	<a href="#">8</a>
<i>Adoxophyes sp.</i>	FTRS-AfL	Neonate larvae	AdorMNPV, PlxyMNPV	<a href="#">8</a>
<i>Agrotis ipsilon</i>	BCIRL/AMCY-AiOV-CLG	Adult ovaries and fat body	PlxyMNPV	<a href="#">9</a>
	BCIRL/AMCY-AiTS-CLG	Adult testes and fat body		<a href="#">9</a>
<i>Amyelois transitella</i>	HCRL-ATO10	Pupal ovaries	AcMNPV	<a href="#">10</a>
	HCRL-ATO20	Pupal ovaries	AcMNPV	<a href="#">10</a>
<i>Anagrapha falcifera</i>	BCIRL/AMCY-AfOV-CLG	Adult ovaries and fat body	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-AfTS-CLG	Adult testes and fat body		<a href="#">9</a>
<i>Antheraea eucalypti</i>	RML-2 subline of Grace's <i>A. eucalypti</i> cells	Pupal ovaries Pupal ovaries	BmNPV	<a href="#">1</a> <a href="#">1</a>
<i>Antheraea pernyi</i>		Pupal ovaries		NP-2
	NISES-AnPe-426	Embryos		<a href="#">11</a>
<i>Antheraea yamamai</i>	NISES-Anya-0611	Pupal ovaries	AnyaNVP	<a href="#">12</a>
<i>Anticarsia gemmatalis</i>	BCIRL/AMCY-AgE-CLG	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-AgOV-CLG1	Adult ovaries and fat body	AgMNPV	<a href="#">9</a>
	BCIRL/AMCY-AgOV-CLG2	Adult ovaries and fat body	AcMNPV, AgMNPV	<a href="#">9</a>
	BCIRL/AMCY-AgOV-CLG3	Adult ovaries and fat body	AcMNPV, AgMNPV	<a href="#">9</a>
	UFL-AG-286	Embryos	AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV	<a href="#">13</a>

<i>Archippus breviplicanus</i>	FTRS-AbL81	Neonate larvae		8
<i>Bombyx mandarina</i>	SES-Bma-O1A	Mature embryos		14
	SES-Bma-O1R	Mature embryos		14
<i>Bombyx mori</i>	Bm-N		BmNPV	NP-3
	Bm5	Larval midguts		15
	Bm-21E-HNU5	Larval ovaries		16
		Embryos	AcMNPV, ArNPV, HearMNPV, PlxyGV	17
	NIV-BM-1296	Larval ovaries	AcMNPV, BmNPV	18
	NIV-BM-197	Pupal ovaries	AcMNPV, BmNPV	18
	SES-Bm-1 30A	Mature embryos		14
	SES-Bm-1 30R	Mature embryos		14
	SES-Bm-e 21A	Mature embryos		14
	SES-Bm-e 21B	Mature embryos		14
	SES-Bm-e 21R	Mature embryos		14
	SES-BoMo-15A	Embryos	BmNPV	19
	SES-BoMo-C129	Embryos	BmNPV	20
	SES-BoMo-JI25	Embryos		20
	SPC-Bm36	Pupal ovaries		NP-4
	SPC-Bm40	Pupal ovaries		NP-4
<i>Buzura suppressaria</i>	WIV-BS-481	Larval hemocytes		NP-5
	WIV-BS-484	Imaginal ovaries	BusuNPV	NP-5
<i>Chilo suppressalis</i>		Larval hemocytes		21
<i>Choristoneura fumiferana</i>	FPMI-CF-1	Midguts		22
	FPMI-CF-2	Midguts		22
	FPMI-CF-3	Midguts		22
	FPMI-CF-203	Midguts		22

(continued)

Table 1 (Continued)

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
	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	<a href="#">23</a>
	IPRI-CF-16	Neonate larvae	CfMNPV	NP-6
	IPRI-CF-16T	Neonate larvae		NP-6
	IPRI-CF-5	Neonate larvae	CfMNPV	NP-6
	IPRI-CF-6	Neonate larvae	CfMNPV	NP-6
	IPRI-CF-8	Neonate larvae	CfMNPV	NP-6
<i>Choristoneura occidentalis</i>		Embryos, neonate larvae and ovaries	CfMNPV	NP-6
<i>Cydia pomonella</i>	CP-1268	Embryos		<a href="#">24</a>
	CP-169	Embryos		<a href="#">24</a>
	CpDW1	Embryos		<a href="#">25</a>
	CpDW2	Embryos		<a href="#">25</a>
	CpDW3	Embryos		<a href="#">25</a>
	CpDW4	Embryos		<a href="#">25</a>
	CpDW5	Embryos		<a href="#">25</a>
	CpDW6	Embryos		<a href="#">25</a>
	CpDW9	Embryos		<a href="#">25</a>
	CpDW10	Embryos		<a href="#">25</a>
	CpDW11	Embryos		<a href="#">25</a>
	CpDW12	Embryos		<a href="#">25</a>

	CpDW13	Embryos		<b>25</b>
	CpDW14	Embryos	CpGV	<b>25</b>
	CpDW15	Embryos	CpGV	<b>25</b>
	200 “primary” cell lines	Embryos and larval hemocytes	ChmuNPV, CpGV	<b>26</b>
	IZD-Cp 4/13			<b>27</b>
	IZD-CP1508	Larval hemocytes		NP-7
	IZD-CP2202	Larval hemocytes		NP-7
	IZD-CP2507	Larval hemocytes		NP-7
	IZD-CP0508	Larval hemocytes		NP-7
<i>Ectropus obliqua</i>	SIE-EO-801	Pupal ovaries		<b>28</b>
	SIE-EO-803	Pupal ovaries		<b>28</b>
<i>Ephestia kuehniella</i>	IPLB-Ekx4T	Embryos	AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV	<b>29</b>
	IPLB-Ekx4V	Embryos	AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, RoMNPV	<b>29</b>
<i>Estigmene acrea</i>	EA1174A (=BTI-EAA)	Larval hemocytes	AcMNPV	<b>30</b>
	EA1174H	Larval hemocytes	AcMNPV	<b>30</b>
<i>Euxoa scandens</i>	IAFEs-1	Ovaries	AcMNPV, BmNPV, GmMNPV, DiwaNPV	<b>31</b>
<i>Galleria mellonella</i>		Ovaries		<b>32</b>
<i>Gnorimoschema operculella</i>	G01-874	Embryos		<b>33</b>
	PTM	Embryos		NP-8

(continued)

**Table 1 (Continued)**

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
<i>Helicoverpa armigera</i>	BCIRL-HA-AM1	Pupal ovaries	AgMNPV	<a href="#">34</a>
	CSIRO-BCIRL-HA1	Ovaries	AcMNPV, HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HA2	Ovaries	HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HA3	Ovaries	HzSNPV	<a href="#">35</a>
	NIV-HA-197	Embryo	AcMNPV, HearSNPV, SpltMNPV	<a href="#">36</a>
<i>Helicoverpa punctigera</i>	CSIRO-BCIRL-HP1	Embryos	AcMNPV, HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HP2	Embryos	AcMNPV, HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HP3	Embryos	AcMNPV, HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HP4	Ovaries	AcMNPV, HzSNPV	<a href="#">35</a>
	CSIRO-BCIRL-HP5	Ovaries	AcMNPV, HzSNPV	<a href="#">35</a>
<i>Helicoverpa zea</i>	BCIRL/AMCY-HzE-CLG1	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG2	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG3	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG5	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG6	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG7	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG8	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL/AMCY-HzE-CLG9	Embryos	AcMNPV	<a href="#">9</a>
	BCIRL-HZ-AM1	Pupal ovaries	HzSNPV, HearSNPV	<a href="#">34</a>
	BCIRL-HZ-AM2	Pupal ovaries	HzSNPV	<a href="#">34</a>
	BCIRL-HZ-AM3	Pupal ovaries	HzSNPV	<a href="#">34</a>
	IMC-HZ-1	Adult ovaries	HzSNPV	<a href="#">37</a>
	IPLB-HZ-1074	Pupal ovaries	HzMNPV	<a href="#">38</a>
	IPLB-HZ-1075	Pupal ovaries and fat body	HzMNPV	<a href="#">38</a>
IPLB-HZ-1079	Fat body	HzMNPV	<a href="#">38</a>	

<i>Heliothis virescens</i>	IPLB-HZ-110	Pupal ovaries	HzMNPV	<b>38</b>
	IPLB-HZ-124Q	Pupal ovaries	HzMNPV	<b>38</b>
	BCIRL/AMCY-HvE-CLG1	Embryos	AcMNPV, AgMNPV, PlxyMNPV	<b>9</b>
	BCIRL/AMCY-HvE-CLG2	Embryos	AcMNPV, AgMNPV PlxyMNPV	<b>9</b>
	BCIRL/AMCY-HvE-CLG3	Embryos	AcMNPV, AgMNPV	<b>9</b>
	BCIRL/AMCY-HvOV-CLG	Adult ovaries	AcMNPV, AgMNPV, PlxyMNPV	<b>9</b>
	BCIRL/AMCY-Hv-TS-GES	Larval testes	AcMNPV	<b>9</b>
	BCIRL-HV-AM1	Pupal ovaries	AcMNPV, AgMNPV, HzSNPV	<b>39</b>
	BCIRL-HV-AM2	Pupal ovaries	AcMNPV, HzSNPV	<b>39</b>
	IPLB-HvE1a	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV	<b>40</b>
	IPLB-HvE1-lt	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, OpMNPV, RoMNPV	<b>40</b>
	IPLB-HvE1s	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV	<b>40</b>
	IPLB-HvE6a	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV	<b>40</b>
	IPLB-HvE6a-lt	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, OpMNPV, PlxyMNPV, RoMNPV	<b>40</b>

(continued)

**Table 1 (Continued)**

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
	IPLB-HvE6s	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, PlxyMNPV, RoMNPV	<b>40</b>
	IPLB-HvE6s-lt	Embryos	AcMNPV, AnfaMNPV, AgMNPV, HzSNPV, RoMNPV	<b>40</b>
	IPLB-HvT1	Larval testicular sheath	AcMNPV, HzSNPV	<b>41</b>
<i>Homona magnanima</i>	FTRS-HmL45	Neonate larvae	PlxyMNPV	<b>8</b>
<i>Hoshinoa longicellana</i>	FTRS-HIL1	Neonate larvae		<b>8</b>
	FTRS-HIL2	Neonate larvae		<b>8</b>
<i>Latoia viridissima</i>		Larval hemolymph	LaviNPV	<b>42</b>
<i>Leucania separata</i>	NIAS-LeSe-11	Larval fat body (female)	AcMNPV	<b>43</b>
<i>Lymantria dispar</i>	IPLB-LD-64	Pupal ovaries	AcMNPV	<b>44</b>
	IPLB-LD-65	Pupal ovaries	LdMNPV	<b>44</b>
	IPLB-LD-66	Pupal ovaries		<b>44</b>
	IPLB-LD-67	Pupal ovaries	LdMNPV	<b>44</b>
	IPLB-LdEG	Embryos	AcMNPV, LdMNPV	<b>41</b>
	IPLB-LdEI	Embryos	AcMNPV, LdMNPV	<b>41</b>
	IPLB-LdEIt	Embryos	AcMNPV, LdMNPV	<b>41</b>
	IPLB-LdEp	Embryos	AcMNPV, LdMNPV	<b>41</b>
	IPLB-LdFB	Larval fat bodies	LdMNPV	<b>41</b>
	IZD-LD1307	Larval testes		NP-9
	IZD-LD1407	Larval testes	AcMNPV	NP-9
	SCLd 135	Ovaries	BmNPV, GmMNPV	NP-9
<i>Malacosoma disstria</i>	IPRI 108	Larval hemocytes	AcMNPV, CfMNPV, LafisoNPV	<b>45</b>

	UMN-MDH-1.	Hemocytes of fifth-instar larvae	MadiNPV	NP-10
<i>Mamestra brassicae</i>	HPB-MB	Adult ovaries	AcMNPV, TnSNPV	NP-11
	IZD-MB0503	Larval hemocytes	AcMNPV, MbMNPV	46
	IZD-MB0504	Larval hemocytes	AcMNPV	46
	IZD-MB1203	Larval ovary and dorsal vessel	AcMNPV	46
	IZD-MB2006	Larval hemocytes		46
	IZD-MB2007	Larval hemocytes		46
	IZD-MB2506	Larval hemocytes	AcMNPV, MbMNPV	46
	MB-H 260	Hemocytes	MbMNPV	47
	MB-H 260	Hemocytes	MbMNPV	47
	MbL-3	Neonate larvae	MbMNPV	48
	MbL-3	Neonate larvae	MbMNPV	48
	NIAS-MaBr-85	Larval fat body (male)	AcMNPV	49
	NIAS-MaBr-92	Larval hemocytes	AcMNPV	50
	NIAS-MaBr-93	Larval hemocytes	AcMNPV	50
	NIAS-MB-19	Pupal ovaries		51
	NIAS-MB-25	Pupal ovaries		51
	NIAS-MB-32	Pupal ovaries		51
	SES-MaBr-1	Larval fat body	AcMNPV	52
	SES-MaBr-2	Larval fat body	AcMNPV	52
	SES-MaBr-3	Larval fat body	AcMNPV	52
SES-MaBr-4	Larval fat body	AcMNPV	52	
SES-MaBr-5	Larval fat body	AcMNPV	52	
<i>Manduca sexta</i>	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		53
	MRRL-CH-2	Embryos		53

(continued)

**Table 1 (Continued)**

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
<i>Mythimna convecta</i>	BPMNU-MyCo-1	Fat body		<b>54</b>
<i>Orgyia leucostigma</i>	IPLB-OIE505A	Embryos	AcMNPV, AnfaMNPV, OpMNPV, OrleNPV, RoMNPV	<b>55</b>
	IPLB-OIE505s	Embryos	OpMNPV, OrleNPV	<b>55</b>
	IPLB-OIE7	Embryos	OpMNPV, OrleNPV	<b>55</b>
	IPRI-OL-12	Neonate larvae	OpMNPV, OpSNPV	<b>56</b>
	IPRI-OL-13	Neonate larvae	OpMNPV, OpSNPV	<b>56</b>
	IPRI-OL-4	Neonate larvae	OpMNPV, OpSNPV	<b>56</b>
	IPRI-OL-9	Neonate larvae	OpMNPV, OpSNPV	<b>56</b>
<i>Ostrinia nubilalis</i>	BCIRL/AMCY-OnFB-GES1	Larval fat body		<b>9</b>
	BCIRL/AMCY-OnFB-GES2	Larval fat body		<b>9</b>
	UMC-OnE	Embryos	AcMNPV, PlxyMNPV	<b>57</b>
<i>Pandemis heparana</i>	FTRS-PhL	Neonate larvae	PlxyMNPV	<b>8</b>
<i>Papilio xuthus</i>	Px-58	Pupal ovaries		<b>58</b>
	Px-64	Pupal ovaries		<b>58</b>
<i>Phthorimaea operculella</i>	ORS-Pop-93	Embryos	AcMNPV, SpliNPV	<b>59</b>
	ORS-Pop-95	Embryos	PhopGV, SpliGV	<b>59</b>
<i>Pieris rapae</i>	BTI-PR10B	Embryos		<b>60</b>
	BTI-PR8A1	Embryos		<b>60</b>
	BTI-PR8A2	Embryos		<b>60</b>
	BTI-PR9A	Embryos		<b>60</b>
	NIAS-PRC-819A	Ovaries		<b>61</b>
	NIAS-PRC-819B	Ovaries		<b>61</b>
	NIAS-PRC-819C	Ovaries		<b>61</b>

<i>Plodia interpunctella</i>	NYAES-PR4A	Embryos		<b>60</b>
	IAL-PID2	Imaginal wing discs	AgMNPV	<b>62</b>
	IPLB-PIE	Embryos		NP-12
	UMN-PIE-1181	Embryos of a malathion-resistant strain		<b>63</b>
<i>Plutella xylostella</i>	BCIRL/AMCY-PxE-CLG	Embryos	AcMNPV, PlxyMNPV	<b>7</b>
	BCIRL/AMCY-PxLP-CLG	Larvae/pupae (whole insects)	AcMNPV, PlxyMNPV	<b>7</b>
	IPLB-PxE1	Embryos	AcMNPV, AnfaMNPV, RoMNPV	NP-12
	IPLB-PxE2	Embryos	AcMNPV, AnfaMNPV, RoMNPV	NP-12
	PX-1187 BCIRL-PX2-HNU3	Embryos	AcMNPV, ArGV, ArNPV, HearMNPV	<b>63</b>
<i>Pseudaletia unipuncta</i>	BTI-Pu-2	Embryos	AcMNPV	<b>66</b>
	BTI-Pu-A7	Embryos	AcMNPV	<b>66</b>
	BTI-Pu-A7S	Embryos	AcMNPV	<b>66</b>
	BTI-Pu-B9	Embryos	AcMNPV	<b>66</b>
	BTI-Pu-M	Embryos	AcMNPV	<b>66</b>
	BTI-Pu-M1B	Embryos	AcMNPV	<b>66</b>
	Several lines	Pupal hemocytes		<b>67</b>
<i>Spilarctia seriatopunctata</i>	NIAS-SpSe-1	Larval fat body (male)		<b>68</b>
<i>Spilosoma imparilis</i>	FRI-SpIm-1229	Larval fat bodies	HycuNPV, SpimNPV	<b>69</b>
<i>Spodoptera exigua</i>	BCIRL/AMCY-SeE-CLG1	Embryos		<b>9</b>
	BCIRL/AMCY-SeE-CLG4	Embryos		
	BCIRL/AMCY-SeE-CLG5	Embryos		<b>9</b>
	Se3FH	Neonate larvae	SeMNPV	<b>70</b>

(continued)

**Table 1 (Continued)**

Species	Designation	Tissue source	Baculovirus infectivity <sup>a</sup>	Ref. <sup>b</sup>
	Se4FH	Neonate larvae	SeMNPV	70
	Se5FH	Neonate larvae	SeMNPV	70
	Se6FHA	Neonate larvae	SeMNPV	70
	Se6FHB	Neonate larvae	SeMNPV	70
	SeHe920-1a	Hemocytes		71
	UCR-SE-1	Neonate larvae	AcMNPV, SeMNPV	72
<i>Spodoptera frugiperda</i>	BCIRL/AMCY-SfTS-GES	Larval testes	AcMNPV, PlxyMNPV	9
	IAL-SFD1	Imaginal wing discs	AcMNPV, AgMNPV, TnSNPV,	62
	IPLB-Sf1254	Pupal ovary	AcMNPV, TnSNPV, SfMNPV	73
	IPLB-Sf21, IPLB-Sf21AE, Sf-9	Pupal ovary	AcMNPV, PlxyMNPV, SfMNPV, SpliNPV, ThorNPV	73
<i>Spodoptera littoralis</i>	HPB-SL	Larvae	AcMNPV, TnSNPV	NP-11
	SPC-SI-48	Pupal ovaries	AcMNPV	74
	SPC-SI-52	Pupal ovaries	AcMNPV	74
	UIV-SL-373	Pupal ovaries	AcMNPV, SeMNPV, SpliNPV, TnSNPV	75
	UIV-SL-573	Pupal ovaries	AcMNPV, SeMNPV, SpliNPV, TnSNPV	75
	UIV-SL-673	Pupal ovaries	AcMNPV, SeMNPV, SpliNPV, TnSNPV	75
<i>Spodoptera litura</i>	IBL-SL1A	Pupal ovaries	SpltNPV	76
	NIV-SU-893	Pupal ovaries		77
	NIV-SU-992	Larval ovaries		78
<i>Spodoptera ornithogalli</i>	BCIRL-503-HNU1	Adult ovaries		79
	BCIRL-504-HNU4	Adult ovaries		79

<i>Trichoplusia ni</i>	BCIRL/AMCY-TnE-CLG1	Embryos	AcMNPV	9
	BCIRL/AMCY-TnE-CLG1MK	Embryos	AcMNPV	9
	BCIRL/AMCY-TnE-CLG2	Embryos	AcMNPV	9
	BCIRL/AMCY-TnE-CLG2MK	Embryos	AcMNPV, PlxyMNPV	9
	BCIRL/AMCY-TnE-CLG3	Embryos	AcMNPV	9
	BCIRL/AMCY-TnTS-GES1	Larval testes	AcMNPV, PlxyMNPV	9
	BCIRL/AMCY-TnTS-GES3	Larval testes	PlxyMNPV	9
	BTI-TN5B1-4 (High Five®)	Embryos	AcMNPV, PlxyMNPV, ThorNPV, TnSNPV	80
	BTI-TN5C1	Embryos		80
	BTI-TN5F2	Embryos		80
	BTI-TN5G2A1	Embryos		80
	BTI-TN5G3	Embryos		80
	BTI-TN5G33	Embryos		80
	IAL-TND1	Imaginal wing discs	AcMNPV, AnfaMNPV, AgMNPV, PlxyMNPV	81
	IPLB-TN-R <sup>2</sup>	3-d-old embryos	AcMNPV, AnfaMNPV, AgMNPV, PlxyMNPV	82
	Several lines	Pupal ovary and fat body	TnSNPV	83
	TN-368	Adult ovaries	AcMNPV, TnSNPV, GmMNPV, AgMNPV, PlxyMNPV	84

<sup>a</sup>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 (7) (see Note 1).

<sup>b</sup>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 BmMNPV but I have been unable to discern the original source (investigator or tissue of origin) of this line (see Note 2).

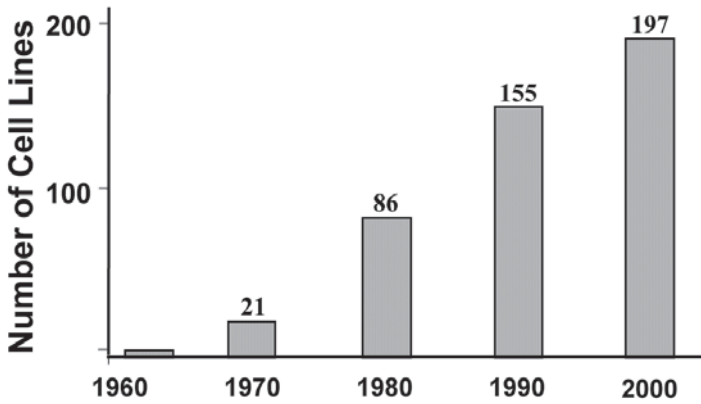


Fig. 1. Number of cell lines reported from Lepidoptera based on the information in [Table 1](#), accumulative by decade.

## 2. Notes

1. [Table 1](#) includes more than 260 cell lines from various lepidopteran species providing a vast supply of material for research on baculoviruses. Although I 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 describing the specific cell line or in one of Hink's compilations [[2–6](#)] or the Granados and Hashimoto review [[7](#)]), approx 60% of these cell lines are known to replicate one or more baculovirus. The designations used in [Table 1](#) are based on the original source of the virus as defined in [Table 2](#). 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.
2. The availability of some of these cell lines is unknown. Although a few insect cell lines are available through repositories (such as the American Type Culture Collection [ATCC], Manassas, VA, or the European Collection of Cell Cultures [ECACC], Health Protection Agency, Porton Down, Salisbury, Wiltshire), researchers interested in the use of most of these cell lines will need to obtain them from other laboratories. If at all possible, this should be from the original source of the cells. Unfortunately, many of the earliest insect cell culturists are no longer active, 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**  
**Baculoviruses Grown in Cell Culture**

Original source and virus genera <sup>a</sup>	Designation
<i>Anagrapha falcifera</i> NPV	AnfaNPV
<i>Antheraea yamamai</i> NPV	AnyaNpV
<i>Anticarsia gemmatalis</i> MNPV	AgMNPV
<i>Artogeia rapae</i> GV	ArGV
<i>Artogeia rapae</i> NPV	ArNPV
<i>Autographa californica</i> MNPV	AcMNPV
<i>Bombyx mori</i> NPV	BmNPV
<i>Buzura suppressaria</i> NPV	BusuNPV
<i>Choristoneura fumiferana</i> MNPV	CfMNPV
<i>Choristoneura murinana</i> NPV	ChmuNPV
<i>Cydia pomonella</i> GV	CpGV
<i>Diapropsis watersii</i> NPV	DiwaNPV
<i>Galleria mellonella</i> MNPV	GmMNPV
<i>Helicoverpa armisgera</i> NPV	HearNPV
<i>Helicoverpa zea</i> SNPV	HzSNPV
<i>Hyphantria cunea</i> NPV	HycuNPV
<i>Lambdina fiscellaaria somniaria</i> NPV	LafiNPV
<i>Latoia viridissima</i> NPV	LaviNPV
<i>Lymantria dispar</i> MNPV	LdMNPV
<i>Malacosoma disstria</i> NPV	MadiNPV
<i>Mamestra brassicae</i> MNPV	MbMNPV
<i>Orgyia leucostigma</i> NPV	OrleNPV
<i>Orgyia pseudotsugata</i> MNPV	OpMNPV
<i>Orgyia pseudotsugata</i> MNPV	OpMNPV
<i>Orgyia pseudotsugata</i> SNPV	OpSNPV
<i>Phthorimaea operculella</i> GV	PhopGV
<i>Plutella xylostella</i> GV	PlxyGV
<i>Plutella xylostella</i> MNPV	PlxyMNPV
<i>Rachiplusia ou</i> MNPV	RoMNPV
<i>Spilosoma imparilis</i> NPV	SpimNPV
<i>Spodoptera exigua</i> MNPV	SeMNPV
<i>Spodoptera frugiperda</i> MNPV	SfMNPV
<i>Spodoptera littoralis</i> GV	SpliGV
<i>Spodoptera littoralis</i> NPV	SpliNPV
<i>Spodoptera litura</i> NPV	SpltNPV
<i>Thysanoplusia orichalcea</i> NPV	ThorNPV
<i>Trichoplusia ni</i> SNPV	TnSNPV

<sup>a</sup>The viruses are NPV, nucleopolyhedrovirus; MNPV, multiple NPVs; SNPV, single NPVs; GV, granulovirus.

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# Lepidopteran Insect Cell Line Isolation from Insect Tissue

Dwight E. Lynn

## Summary

This chapter describes procedures for initiating new cell lines from lepidopteran larval tissues. 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; tissue sources; dissection techniques; culture medium.

## 1. Introduction

As shown in Chapter 6, established cell lines from Lepidoptera are widely available, with over 260 lines from more than 60 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 the author has found effective for initiating new cell lines. The author has previously described methods for initiating cultures from embryos (*1*) but will primarily concentrate on larval tissue in this chapter with some notes describing 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  $\mu\text{g}/\text{mL}$  gentamicin sulfate (*see Note 2*).

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
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2. Divalent ion-free phosphate buffered saline for enzyme dissociation: 800 mg NaCl, 20 mg  $\text{KH}_2\text{PO}_4$ , 20 mg KCl, 150 mg  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 23 mg  $\text{Na}_2\text{EDTA}$  in demineralized water to 100 mL.
3. VMF Trypsin (virus and mycoplasma-free; cell culture tested).
4. 70% Ethanol.
5. Sterile (autoclaved) demineralized water.

## 2.2. Tissue Culture Labware

1. 35-mm Tissue culture Petri dishes.
2. 12.5- or 25-cm<sup>2</sup> Tissue culture flasks.

## 2.3. Equipment

1. Laminar flow clean bench (*see Note 3*).
2. Dissecting tools, including two or more pairs of jeweler's forceps, microscalpel, microscissors (*see Note 4*).
3. Insect pins.
4. Small jar with a layer of cotton in the bottom containing 95% ethanol (*see Note 5*).
5. Alcohol lamp with wind screen (*see Note 6*).
6. Sterile (autoclaved) glass Petri dish containing 4–5 mm paraffin (= wax dish).
7. Stereo dissecting microscope and light source.
8. 1-mL Sterile pipets.
9. 250- $\mu\text{L}$  Pipettor.
10. Sterile pipet tips.
11. Plastic box, approx  $5 \times 6 \times 2$  in. with air-tight lid.

## 3. Methods

### 3.1. Disinfection

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

1. Submerge the insect in 70% ethanol for 5–20 min. The shorter time should be used with particularly fragile (thin-cuticled) insects and the longer time if the insect's typical environment is particularly dirty (*see Note 7*).
2. Rinse in 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 submerged in this manner will result in a buildup of  $\text{CO}_2$  from their natural respiration and this will act as an anesthetic to keep them immobile during 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

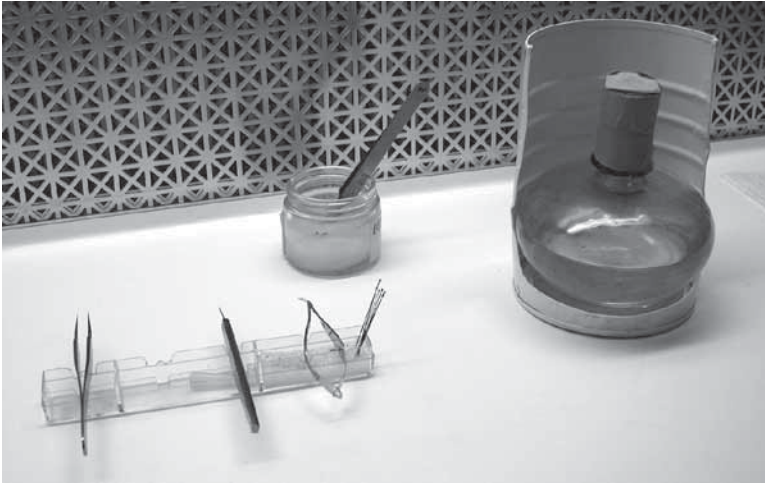


Fig. 1. Typical arrangement of equipment in the clean bench for dissections. The jar in the center contains ethanol and a small layer of cotton in the bottom. The dissecting tools are placed in the jar for at least 5 min, after which the alcohol is ignited by passing the tip over the alcohol lamp's flame (*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.

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 media 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, body cut the cuticle from tail to the first thoracic segment, taking care to not pierce the gut.
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**).

### 3.2.1. Internal Morphology

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

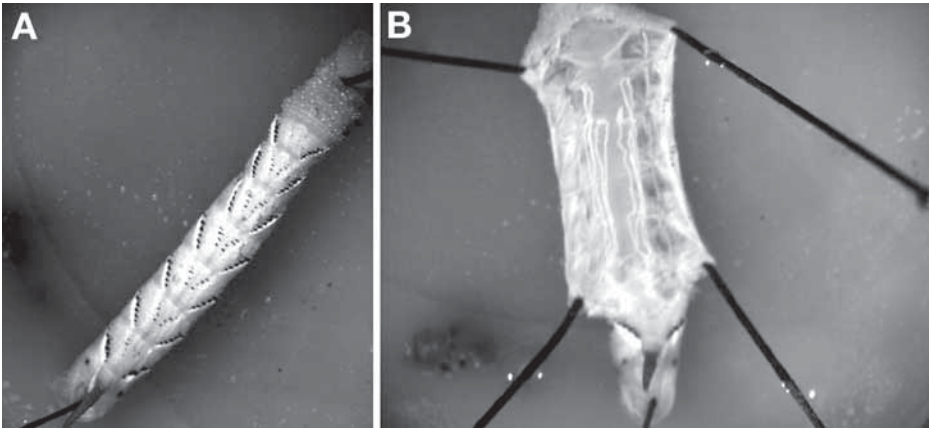


Fig. 2. A fourth instar *Manduca sexta* larva pinned in a wax dish containing tissue culture medium. (A) Larva before first incision. (B) Larva with cuticle pinned to each side to expose internal organs and tissues.

#### 3.2.1.1. DIGESTIVE TRACT

Because larval Lepidoptera are essentially eating machines, the most obvious structure is the digestive tract (Figs. 3–5, 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 (2) (see Note 10), you should be especially cautious to avoid rupturing it.

#### 3.2.1.2. MALPHIGIAN TUBULES

Loosely connected to the midgut are the Malphigian tubules (Figs. 3–5, 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.

#### 3.2.1.3. 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 owing to the refraction of the air they contain.

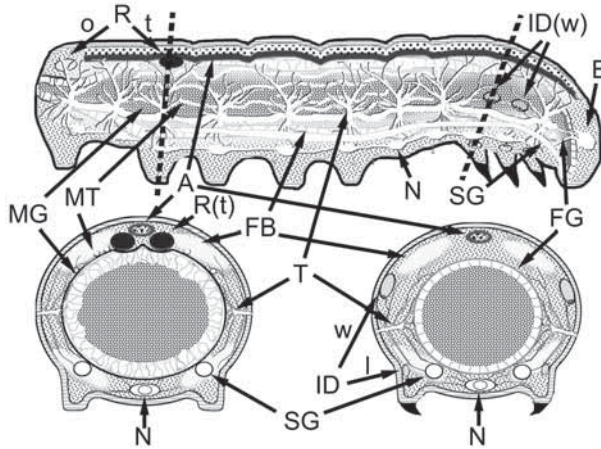


Fig. 3. Diagrams of lepidopteran larva showing the locations of many tissues. (Top) Lateral view; (Lower right) cross section through thorax; (Lower left) cross section through abdomen. R, reproductive tissue (o, ovaries; t, testis); ID, imaginal discs (w, wings; l, legs); B, brain; MG, midgut, MT, Malphigian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

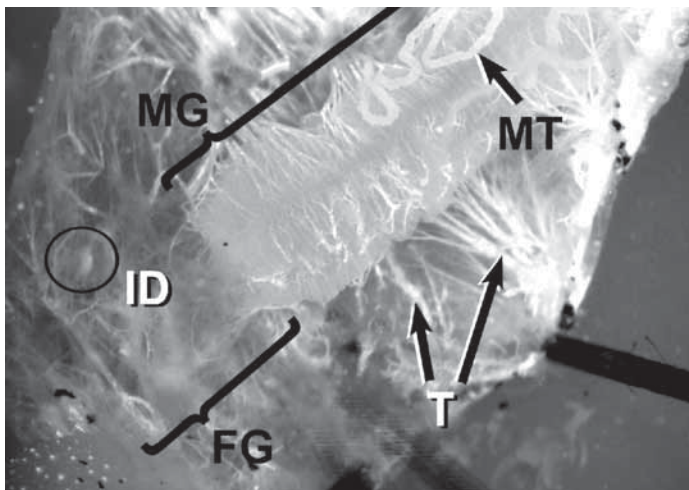


Fig. 4. Thoracic and anterior abdomen of *Manduca sexta* larva. ID, imaginal discs; MG, midgut, MT, Malphigian tubules; FG, foregut; T, tracheoles. The circle is the location of one of the imaginal discs. At this stage in the dissection, the lateral muscles largely obscure it, but the faint white mass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

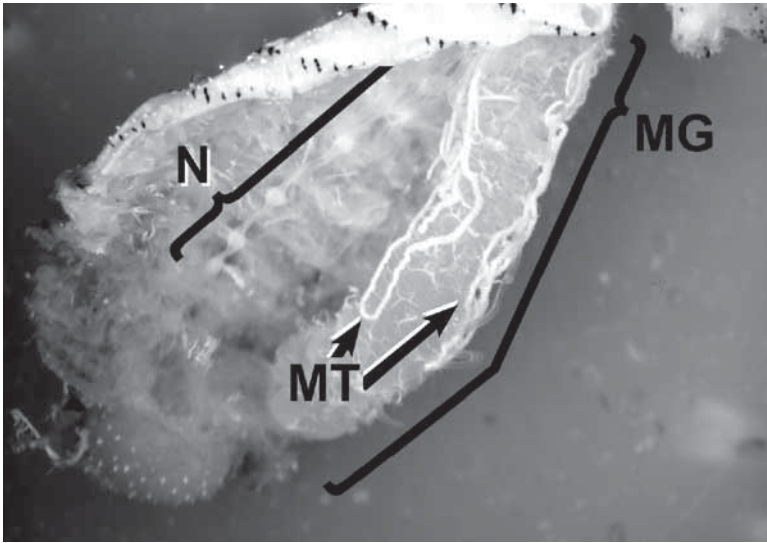


Fig. 5. Thoracic and anterior abdomen of *Manduca sexta* larva showing a large portion of the abdomen with the midgut moved to the side revealing the ventral nerve cord (N). MG, midgut; MT, malpighian tubules.

#### 3.2.1.4. 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.

#### 3.2.1.5. 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.

#### 3.2.1.6. 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. (**Figs. 3** and **5**, SG).

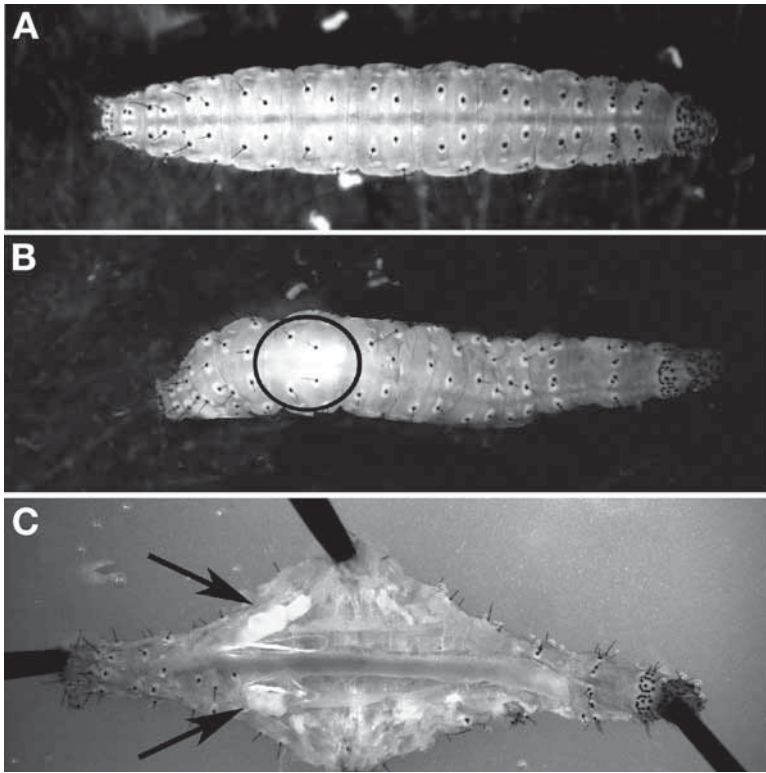


Fig. 6. *Plutella xylostella* larvae. (A) Female last instar, (B) male last instar. The circle indicates the location of the testes, (C) dissected male larva, arrows indicate testes.

### 3.2.1.7. 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. 3A). 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.

### 3.2.1.8. REPRODUCTIVE ORGANS

The reproductive organs will be in the abdomen, generally dorsal to the hindgut (ovaries) or midgut (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 (Figs. 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.

### 3.2.1.9. IMAGINAL DISCS

More challenging than 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 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 (3), but the tissues are more tightly connected 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 (4,5). Imaginal disc tissues that are destined to become eyes, antennae, legs, and wings have been identified, but the wing discs are the easiest to locate and excise in Lepidoptera. Because 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, 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 owing to the refraction of the air they contain and these can be used to help locate the discs (Figs. 4 and 7).

### 3.2.2. Initiation of Primary Cultures

1. When the tissue of choice has been identified (*see Note 12*), 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*).

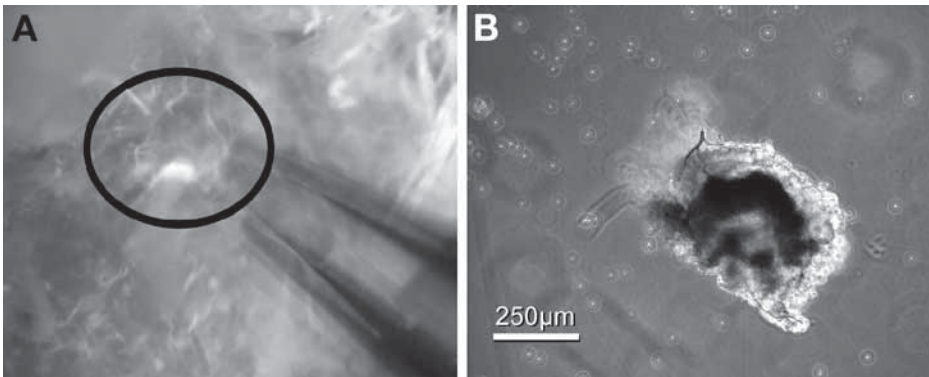


Fig. 7. Wing imaginal discs of *Manduca sexta*. (A) Disc (circled) being slightly lifted from the cuticle with forceps. The white spot is the tracheoles. (B) Wing imaginal disc in culture. The tracheoles, which are white in the other micrographs with reflective light appear as a dark mass under the transmitted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

3. Once the tissue extractions are complete, transfer them to a new culture dish containing a standing drop (100–150  $\mu\text{L}$ ) of medium or enzyme buffer. At this point, the tissues will each have a distinctive appearance as seen in **Figs. 7–9**.
4. Use a microsurgical scalpel to cut the tissue into small fragments, or enzymatically dissociate 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.
6. After completing the manipulations, the dish should be sealed by stretching a thin strip of Parafilm® (~(approx  $8 \times 100$  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.

### 3.3. Initial Maintenance

1. One to 2 d 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- to 10-d intervals, adding 0.5 mL fresh medium to all healthy cultures. This routine should be continued until the dish contains sufficient cells for subcultivation.

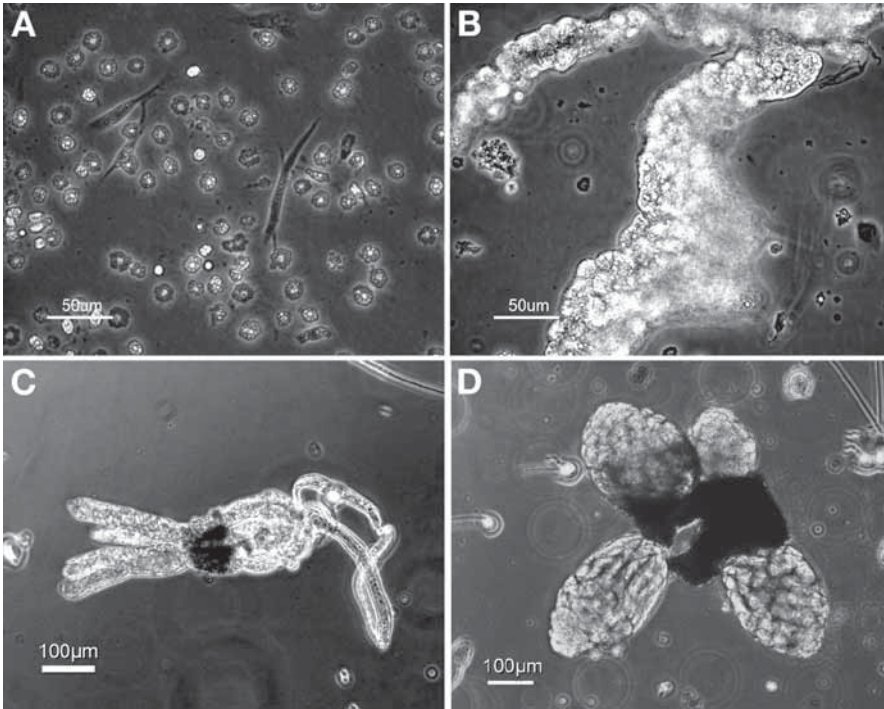


Fig. 8. Larval cells and tissues in primary cultures. Each of these tissues has been used for establishing cell lines. (A) Hemocytes in a dissecting dish (*see Note 14*), (B) fat body, (C) ovaries, (D) testis (the sheath has been ruptured showing four follicular bundles).

4. If the volume of medium reaches approx 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 50g for 5 min and the pellet resuspended into 0.5 mL fresh medium and 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 because 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.

### 3.4. 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. The author prefers to

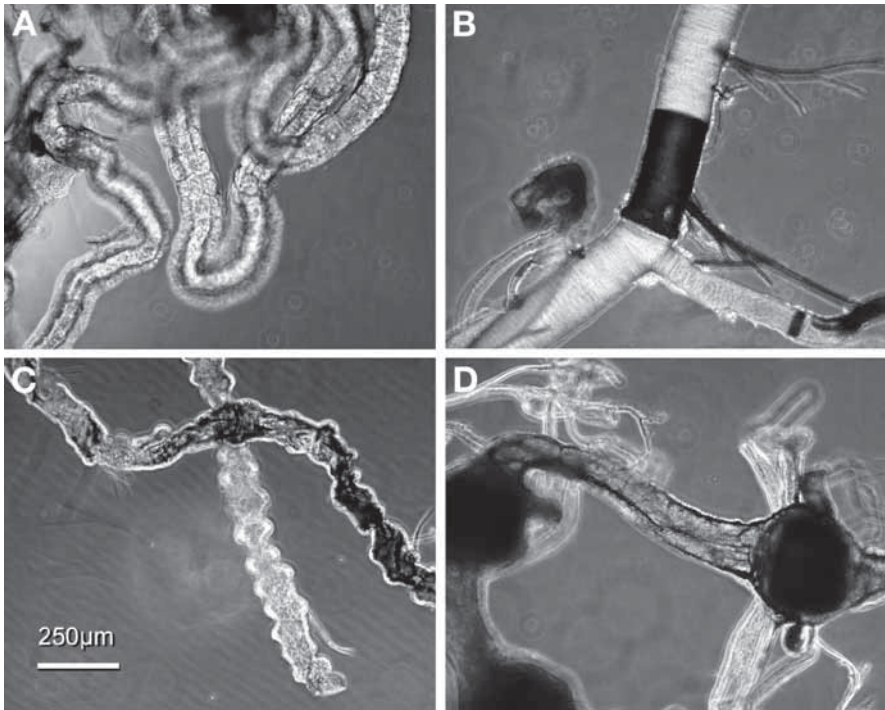


Fig. 9. Additional larval tissues in primary cultures. The tissues shown in this figure have not been used for establishing cell lines, although some have been used in primary tissue and cell culture studies. (A) Salivary gland, (B) tracheoles, (C) malpighian tubules, (D) ventral nerve cord.

use a small flask (some manufacturers make a 12.5-cm<sup>2</sup> version) for the first daughter cultures because these can be tightly capped to reduce the chance of contamination and dehydration.

- 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.

### 3.5. Additional Subcultivations

If there are a substantial number of attached cells remaining, then another subculture may be performed within 1 or 2 wk. For the second split, a more vigorous method is typically used for detaching the cells.

#### 3.5.1. Cultures in a Flask

- Chill the culture for 20 min at 4°C.
- 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.

### 3.5.2. For 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 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- to 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<sup>2</sup> 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 approx 2.0 mL in a 12.5-cm<sup>2</sup> or 4.0 mL in a 25-cm<sup>2</sup> 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).

## 3.6. Strain Selection

### 3.6.1. 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. If two cells exist in a culture, one of which completes the cell cycle in half the time of the other, the slower cell type will be outnumbered 1000 to 1 within 10 of its divisions and 1 million to 1 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.

### 3.6.2. Attachment

Other features besides growth rates can influence the distribution of cell types in the culture, as well. For example, suppose in the previous example the faster growing cells were much more adherent to the culture flask so 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 nonattached 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, so as to maintain the widest diversity of cell types until some indication of which cell type is useful in the specific application for which it is needed. 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, the author's medium of choice was TC-100 (originally described as BML-TC/10) (6) with some additional supplements (peptones, additional vitamins, and trace minerals as described previously [1] but in recent efforts, both the author [7] and other researchers [8] have used one of 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 although gene homologs 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, the author expounds 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 U penicillin and 0.1 mg/mL streptomycin culture medium) to reduce contamination in primary insect cultures. Antifungal agents are particularly toxic to insect cells (9), probably because the biochemical pathways they target also occur in insects and thus the author does 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 because 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. The author has 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, 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, a longer disinfection with 70% ethanol can be used or 0.05%  $\text{HgCl}_2$  in 70% ethanol can be used instead. If the tissues of interest are embryos, 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 making 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 because 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, whereas holding them with the tips directly below your hand will result in the heat of the flame being directly below your fingers. Because 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 describe 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. (10) indicate they could be extremely useful as indicator cells because in some virus/insect systems, they are responsible for initially spreading the infection.
12. As discussed in Chapter 6, a wide variety of tissues have been used for establishing lepidopteran cell lines. Although not discussed in detail in this chapter, embryos are a common source of cell lines and one that the author has favored over the years. Although less precise than the larval tissues discussed in this chapter, progenitors for every cell type exists in the embryos and many of them are already in active states of cell division. The author's usual method for initiating primary cultures from embryos is to disinfect eggs (approximately half way through development to a first instar larva) in 70% ethanol followed by two sterile demineralized water rinses and then a transfer to culture medium containing antibiotics as described above. Squeezing one end with the microforceps ruptures the chorion or it can be cut with a microscalpel. Twenty to 50 embryos are then transferred to a standing drop (100–150  $\mu\text{L}$ ) of fresh medium with antibiotics and treated in the same manner as the specific tissues in **Subheadings 3.2.–3.4.**
13. Although 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. 8A).
14. Many insect tissues have a wound response to cuts and will initiate cell division spontaneously. However, treatment with a mild concentration of trypsin, but not long enough to completely disassociate the tissue to single cells may improve migration and growth of cells from the tissue explants. Use 50  $\mu\text{g}/\text{mL}$  trypsin in the divalent-free phosphate buffered saline. Treat the tissue for 5–20 min and then replace the trypsin solution with medium (100–150  $\mu\text{L}$  in a standing drop) containing 50  $\mu\text{g}/\text{mL}$  gentamicin. Two forceps are then used to tear it 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 you let the cells become too dense, there is a potential that they will deplete the nutrients in the medium and stop growth. 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, resulting in very low cell densities, these growth factors will disperse into the medium causing a reduced or even inhibition of cell growth.

## Acknowledgments

The author thanks Dr. Robert Farrar and Ms. Lynda Liska for providing insects used in these studies. 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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## Development of Serum-Free Media for Lepidopteran Insect Cell Lines

Spiros N. Agathos

### Summary

Lepidopteran insect cell culture technology has progressed to the point of becoming an essential part of one of the most successful eukaryotic expression systems and is increasingly used industrially on a large scale. Therefore, there is a constant need for convenient and low-cost culture media capable of supporting good insect cell growth and ensuring high yield of baculovirus as well as the strong expression of recombinant proteins. Vertebrate sera or invertebrate hemolymph were essential supplements in first-generation insect cell media. These supplements, however, are cumbersome and expensive for routine large-scale culture; thus, their use is now circumvented by substituting the essential growth factors present in these supplements with serum-free substances. Such non-serum supplements are typically of non-animal origin and include protein hydrolysates, lipid emulsions, and specialized substances (e.g., surfactants and shear damage protecting chemicals). These supplements need to complement the defined, synthetic basal medium to ensure that the fundamental nutritional needs of the cells are satisfied. Although there is a significant number of proprietary serum-free and low-protein or protein-free media on the market, the lack of information concerning their detailed composition is a drawback in their adoption for different applications, including their adaptation to the metabolic and kinetic analysis and monitoring of a given insect cell based bioprocess. Hence, there is wide appeal for formulating serum-free media based on a rational assessment of the metabolic requirements of the lepidopteran cells during both the growth and the production phases. Techniques such as statistical experimental design and genetic algorithms adapted to the cellular behavior and the bioreactor operation mode (batch, fed-batch, or perfusion) permit the formulation of versatile serum- and protein-free media. These techniques are illustrated with recent developments of serum-free media for the cultivation of commercially important *Spodoptera frugiperda* and *Trichoplusia ni* cell lines.

**Key Words:** Culture media; serum; serum-free; animal-free; protein-free; protein hydrolysates; factorial experimental design; genetic algorithm.

From: *Methods in Molecular Biology*, vol. 388: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

## 1. Introduction

Insect cell lines derived from lepidopteran species were first used on a large scale for producing wild-type baculovirus preparations as biopesticides against harmful insects. However, the current explosive interest in insect cell culture technology as a major biomanufacturing platform (1,2) is the result of the development of the baculovirus expression vector system (BEVS) in the early 1980s (3,4). The majority of the expression vectors are based on the *Autographa californica* multiple nucleopolyhedrovirus. Most of the work on insect cell culture, including the applications of the BEVS, has been carried out with cell lines primarily from the *Spodoptera frugiperda* (Fall armyworm) species. The first line to be widely used was Sf-21 (5) and originated from *S. frugiperda* ovaries. Sf-9, a clone of Sf-21 with improved growth and production characteristics, progressively replaced it in research and production fields. Another cell line, Tn-368, was derived from ovarian tissues of the cabbage looper, *Trichoplusia ni* (6), but its significance has declined in favor of a far more promising line from the same lepidopteran insect, BTI-Tn-5B1-4 (Tn-5). This cell line is a clonal isolate of the BTI-Tn-5B1-28 cell line originally isolated from *T. ni* embryos (7,8). This cell line has been shown to generally have a superior capacity for secreted glycoprotein production compared to Sf cell lines (9). The Tn-5 cell line, commercially available from Invitrogen as High Five™ cells, is now routinely used in the BEVS as seen by the steady rise in relevant publications and media formulations (especially serum-free) developed for their cultivation.

The growth of insect cells, just like that of all animal cells in vitro, requires an environment favoring their proliferation outside their tissue of origin. Early insect cell culture involved cultivating them in basal mixtures of vitamins, amino acids, carbohydrates, and salts supplemented with a poorly defined biological fluid such as serum or hemolymph (the circulatory fluid of invertebrates). Serum rapidly became the preferred supplement for the culture of insect cell lines. Among its multiple benefits, it provides growth factors, proteins with detoxifying and antioxidant effects, carrier proteins, and protease inhibitors. However, the use of serum in cell culture has several drawbacks, including high cost, lot-to-lot variability, limited or unreliable supply, and concerns about the presence of adventitious agents or contaminants. Furthermore, its high protein content can interfere with the purification process of recombinant proteins. These concerns have stimulated interest in developing serum-free, protein-free, and, ideally, chemically defined media. Despite the attractiveness of serum-free culture, medium development can be tedious and costly. The cost factor is more pronounced when serum- and protein-free media devoid of any component of animal origin are required or when media need to be supplemented with expensive growth factors, attachment factors, and protease inhibitors.

Insect cells have traditionally been cultured in basal media, such as Grace's medium (10), TNM-FH (6), or TC-100 (11), supplemented with 5 or 10% fetal bovine serum. In the last several years serum-free media are becoming more dominant in insect cell culture. Since the 1980s the formulation of IPL-41 (12) has provided a reliable basal medium for serum-free culture. Its potential for large-scale cultivation (e.g., in agitated and airlift bioreactors) was proven upon supplementation with 4 g/L yeastolate (ultrafiltered yeast extract obtained by autolysis of yeast biomass) and a lipid mixture emulsified in Pluronic® F-68 (13). Another serum-free medium shown to support higher cell densities and protein production than serum-supplemented media, called SFM-LP, is based on TNM-FH supplemented with Pluronic F-68 (0.1 w/v%) and Ex-Cyte VLE, a low protein water-soluble source of lipids (14). In the meantime, the involvement of biochemical companies in insect cell culture has been yielding an increasing number of commercial serum-free media. Several media, e.g., Sf-900 II (15) and Express Five™ SFM (16) from Invitrogen (GIBCO™ Life Technologies, <http://www.invitrogen.com>), Insect-XPRESS™ from Cambrex (<http://www.cambrex.com>) (now Lonza), EX-Cell 400 (17), EX-Cell 405 and EX-Cell 420 from JRH Biosciences (<http://www.jrhbio.com>), and HyQ SFX-Insect™ (18) from Hyclone (<http://www.hyclone.com>), have been developed over the last decade and are used routinely for insect cell cultivation. These media, although able to support high cell densities and protein titers, are generally expensive, cell-line specific and of proprietary composition. A listing of most serum-free (and often protein-free) media currently available for insect cell culture has been compiled in Table 1. In response to increasing regulatory scrutiny, a current trend in media design is the elimination not only of serum but also of animal-derived components. Thus, several of the serum-free media listed in Table 1 are chemically defined and entirely animal component-free. The lack of information concerning the detailed composition of commercial serum-free media is often a limitation in their adoption for different applications, including their adaptation to the metabolic and kinetic analysis and monitoring of a given insect cell based bioprocess. Hence, there is a need for formulating serum-free media based on a rational assessment of the metabolic requirements of the lepidopteran cells during both the growth and production phases.

A promising set of supplements designed to replace serum for the large-scale, serum-free culture of animal cells are protein hydrolysates. These substances, also known as peptones, are complex mixtures of oligopeptides, polypeptides, and amino acids that are produced by enzymatic or chemical digestion of casein, albumin, plant or animal tissues, or yeast cells. Primatone RL (an enzymatic digest of animal tissue) is a cost-effective supplement, which, under serum-free conditions, is able to prolong the stationary phase by delay-

**Table 1**  
**Commercial and Nonproprietary Serum-Free Media for Lepidopteran Insect Cell Cultivation<sup>a</sup>**

Product	Supplier or reference	Composition	Application	Cells
BIOINSECT-1	BIOLOGICAL INDUSTRIES <a href="http://www.bioind.com">http://www.bioind.com</a>	Serum-free medium	Culture of lepidopteran insect cells	Sf-9, Sf-21
Insect-XPRESS™	CAMBREX <a href="http://www.cambrex.net">http://www.cambrex.net</a>	Serum- and protein-free medium, contains L-glutamine	Designed to support the growth of invertebrate cell lines derived from <i>Spodoptera frugiperda</i> (Sf), under attachment dependent or attachment independent conditions. Supports production of recombinant proteins by cells infected with viral vectors such as baculovirus expression vector system (BEVS)	Sf-9 and Sf-21 grown as adherent monolayers or as suspension cultures
Serum-free media	CHEMICON International <a href="http://www.chemicon.com/">http://www.chemicon.com/</a>	Serum-free, customized media	Developed for growth of insect cell lines	Insect cell lines
ESF 921	EXPRESSION SYSTEMS <a href="http://expressionsystems.com/">http://expressionsystems.com/</a>	Serum-, protein-free media. Unknown composition	Developed for transfection of insect cell lines	Insect cell lines
ESF AF		Serum-, protein-free media, unknown composition, devoid of animal products	Developed for transfection of insect cell lines	Insect cell lines

MED-10002	GENTAUR <a href="http://www.gentaur.com">http://www.gentaur.com</a>	Serum-free medium of proprietary composition, contains high-density lipids, shear protectants, and optimum levels of glucose and glutamine	Optimized for high-density growth of <i>S. frugiperda</i> (Sf-9, Sf-21) and <i>Trichoplusia ni</i> (incl. Tn-5) cells for the production of recombinant proteins.	Sf-9, Sf-21 Tn-368, Tn-5
MED-10003		Serum-, methionine-free insect culture medium of proprietary composition	Formulated for baculovirus-based expression in <i>S. frugiperda</i> (Sf-9, Sf-21) and <i>T. ni</i> (incl. Tn-5) cells	Sf-9, Sf-21 Tn-368, Tn-5
Express Five™ SFM	GIBCO™ INVITROGEN CORPORATION <a href="http://www.invitrogen.com">http://www.invitrogen.com</a>	Protein- and serum-free complete medium	Growth and maintenance of Tn-5 cells in the BEVS for adherent or suspension cultures, large-scale production of recombinant protein expressed by BEVS	Tn-5
Sf-900 II SFM		Protein- and serum-free medium	Growth and maintenance of cells used for BEVS for adherent or suspension culture, large-scale production of recombinant protein expressed by BEVS	Sf-9, Sf-21 Tn-368
HyQ CCM3™	HYCLONE <a href="http://www.hyclone.com">http://www.hyclone.com</a>	Serum-free complete medium, contains glutamine, without phenol red	Supports growth of Sf-9 cells	Sf-9

(continued)

Table 1 (Continued)

Product	Supplier or reference	Composition	Application	Cells
HyQ SFX-Insect™		Protein-free medium. Contains Pluronic® F-68, devoid of animal products	Supports the growth of multiple insect cell lines and production of a variety of recombinant proteins	Sf-9, Sf-21 Tn-5 D.mel 2 ( <i>Drosophila</i> )
IPL-41 PF		Protein-free basal medium	Used for optimisation of serum-free conditions of insect cell maintenance and protein expression	Various lepidopteran insect cells
IS-BAC	IRVINE SCIENTIFIC <a href="http://www.irvinesci.com">http://www.irvinesci.com</a>	Serum- and protein-free medium	Supports long-term growth of insect cells for production of insect virus and recombinant DNA proteins	Sf-9, Sf-21 Tn-5
EX-CELL™ 400	JRH Biosciences <a href="http://www.jrhbio.com">http://www.jrhbio.com</a>	Protein- and serum-free medium, hydrolysate source yeast, without hypoxanthine and thymidine	Designed for growth of cell lines from <i>S. frugiperda</i> , can be used to propagate insect cells for expressing recombinant products, can be used in both suspension and adherent culture systems	<i>S. frugiperda</i> (including Sf-9 and Sf-21)
EX-CELL™ 405		Protein- and serum-free medium, hydrolysate source yeast, without hypoxanthine and thymidine, devoid of animal products	Optimized for the nutrient and environmental needs of <i>T. ni</i> (Tn-5), can be used for expressing recombinant products using the BEVS, can	Tn-5 cells

EX-CELL™ 420		Protein- and serum-free medium, contains L-glutamine, Pluronic F-68, and glucose, hydrolysate source yeast, without hypoxanthine and thymidine	be used in both suspension and adherent culture systems Optimised for growth of Sf-9 and Sf-21 cells, can be used in both suspension and adherent culture systems	Sf-9, Sf-21 Schneider S2 ( <i>Drosophila</i> )
Serum-free insect Virus Production Medium	MP BIOMEDICALS <a href="http://www.mpbio.com">http://www.mpbio.com</a> (formerly ICN Biomedicals)	Complete serum-free medium, with L-glutamine	Formulated for production of insect viruses	Insect cell virus production
Insectagro FIVE™	MEDIATECH INC <a href="http://www.cellgro.com">http://www.cellgro.com</a> or <a href="http://www.krackeler.com">http://www.krackeler.com</a>	Serum- and protein-free medium with L-glutamine	For growth of Tn-5 cells	Tn-5
Insectagro Sf9™		Serum- and protein-free medium with L-glutamine	For growth of Sf9 cells	Sf-9
BacVector™ Insect Cell Medium	MERCK BIOSCIENCES <a href="http://merckbiosciences.de">http://merckbiosciences.de</a>	Serum-free medium	For culture of insect cells to high densities	Sf-9 Ea-4
Insect Express™ High Five	PAA <a href="http://www.paa.at">http://www.paa.at</a>	Protein-free and chemically defined medium	Developed for the culture of and recombinant protein expression in Tn-5 cells	Insect cells, including <i>Trichoplusia</i> cells
Insect Express™ Sf9-S2		Serum- and protein-free medium	For the adherent or suspension culture of insect cells in various culture systems, developed for baculovirus expression systems and use with BEVS	Sf-9 Schneider S2 ( <i>Drosophila</i> )

(continued)

**Table 1 (Continued)**

Product	Supplier or reference	Composition	Application	Cells
CYTOMAX	PGC SCIENTIFICS <a href="http://www.pgscientific.com/">http://www.pgscientific.com/</a>	Serum-free supplement for use with basal medium	For the cultivation of a broad spectrum of mammalian and insect cells in a number of growth cell systems, including hollow fibre and bioreactors, suitable for adherent and suspension culture, suitable for hybridoma cultures, HeLa/vaccinia vector systems and protein expression	Broad spectrum including hybridomas, HeLa, vaccinia, insect cells
162 IP301 BaculoExpress ICM	PROMOCELL <a href="http://www.promocell.com">http://www.promocell.com</a> or BIOCONCEPT AG <a href="http://www.bioconcept.ch">http://www.bioconcept.ch</a>	Chemically defined media, serum-free media, requires supplementation with lipids and Pluronic F-68	For culture of insect cell lines for production of recombinant proteins in the BEVS	Sf-9, Sf-21
SF-1 BaculoExpress ICM		Serum-free media, requires supplementation with lipids and Pluronic F-68	For culture of insect cell lines for production of recombinant proteins in the BEVS	Sf-9, Sf-21
SF-3 BaculoExpress ICM		Serum- and protein-free medium	Ready to use for cultivation of insect cells	Sf-9, Sf-21 Tn-5 D.Mel-2 ( <i>Drosophila</i> )
SF-4 BaculoExpress ICM		Proprietary formulation, serum-free and requires no supplementation	Used to grow various <i>S. frugiperda</i> (Sf-9, Sf-21), <i>T. ni</i> (Tn-5) and <i>Drosophila melanogaster</i> (D.Mel-2) cells	Insect cell culture

IPL-41 insect medium	SIGMA-ALDRICH Co. <a href="http://www.sigma-aldrich.com">http://www.sigma-aldrich.com</a>	Serum-free medium containing L-glutamine and sodium bicarbonate	Can be used unsupplemented for BEVS driven protein expression	Insect cells
Serum-free medium-1 protein-free		Serum- and protein-free complete medium with L-glutamine and sodium bicarbonate, based on IPL-41 medium originally developed at USDA, contains inorganic salts, amino acids, vitamins, other organic compounds, and trace elements	Developed for production of recombinant proteins	Insect cells
Serum-free medium-2 low-protein		Serum-free and low protein complete medium with L-glutamine and sodium bicarbonate, based on IPL-41 medium originally developed at USDA, contains inorganic salts, amino acids, vitamins, other organic compounds, and trace elements	Developed for production of recombinant proteins	Insect cells

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*(continued)*

**Table 1 (Continued)**

Product	Supplier or reference	Composition	Application	Cells
TiterHigh™ Sf Insect Medium		Serum-, protein-free medium, complete, requires no supplementation, contains inorganic salts, amino acids, fatty acids, and other organic compounds, devoid of animal products	Designed for high viable cell densities with the Sf-9 and Sf-21 lines of <i>S. frugiperda</i> , optimized for high viral titers of wild type AcMNPV and for high levels of recombinant protein production with the BEVS	Sf-9, Sf-21
164 SFM-LP	14	Serum-free medium, based on TNM-FH basal medium (without serum), supplemented with Ex-Cyte VLE and Pluronic F-68 polyol	Developed for growth of <i>S. frugiperda</i> (Sf-9) and <i>T. ni</i> (TN-368) cultivated in suspension	Sf-9 Tn-36
SF-1	20,21	Serum- and protein-free low-cost medium with L-glutamine and glucose, based on IPL-41 basal medium, supplemented with yeastolate ultrafiltrate, Primatone RL, lactalbumin and Pluronic F-68—lipid mixture	Optimized for growth of <i>S. frugiperda</i> (Sf-9), <i>T. ni</i> (Tn-5) and <i>D. melanogaster</i> (SL-3) cells in large-scale suspension for the production of recombinant proteins	Sf-9 Tn-5 SL-3 ( <i>Drosophila</i> )

KDM-10	45	Serum-free medium	Designed for metabolic studies on <i>S. frugiperda</i> (Sf-9) cells	Sf-9
Serum-free medium	48	Serum-free medium	Designed for metabolic studies on <i>S. frugiperda</i> (Sf-9) cells	Sf-9
ISYL	22	Serum- and protein-free low-cost medium with glucose, based on IPL-41 basal medium, supplemented with yeastolate ultrafiltrate, soy protein hydrolysate, dextran sulfate, and Pluronic F-68—lipid mixture, devoid of animal products	Developed for optimal growth of <i>T. ni</i> (Tn-5) cells in suspension for the production of recombinant proteins	Tn-5
YPR	19	Serum- and protein-free low-cost medium with L-glutamine and glucose, based on IPL-41 basal medium, supplemented with yeastolate ultrafiltrate, Primatone RL (or soy protein hydrolysate for a medium devoid of animal products) and Pluronic F-68—lipid mixture	Rationally designed for optimal growth of <i>S. frugiperda</i> (Sf-9, Sf-21) and <i>T. ni</i> (Tn-5) cells in suspension for the production of recombinant proteins	Sf-9, Sf-21 Tn-5

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<sup>a</sup>Adapted and expanded from a compilation by Focus on Alternatives, <http://www.focusonalternatives.org.uk>, (July 2005 version).

ing the apoptotic death of both Sf-9 and Tn-5 insect cells (19). These cells grown in either shake flasks or in a 1-L stirred bioreactor in our own Primatone and yeastolate-containing serum-free medium, named YPR, have prolonged stationary phases of 3–4 d at cell concentrations varying from 3 to  $7 \times 10^6$  cells/mL (19) and unpublished results). Primatone is also one of the major constituents of the SF-1 medium, a low-cost serum-free medium that supports Sf-9 cell growth to  $10^7$  cells/mL in spinner flasks and in a 23-L airlift bioreactor (20). Other insect cell lines, including *Drosophila melanogaster* SL-2 and SL-3 and *T. ni* Tn-5, have been successfully adapted to this medium (21). A soy protein hydrolysate (Hy-Soy) is an essential component (at a concentration of 4 g/L) of the ISYL medium for insect cells, a serum-free formulation based on IPL-41 (22). This medium compared well with EX-Cell 405 in terms of cell growth (maximal cell density of about  $6 \times 10^6$  cells/mL) and volumetric production level of recombinant secreted alkaline phosphatase (about 60–70 mg/L) for the Tn-5 cell line. In our own group we also observed immediate adaptation and good growth over several passages for Tn-5 cells transferred from YPR medium (19) to medium containing SE50MAF, a soy protein hydrolysate, instead of Primatone RL (unpublished results). Thus, soy protein hydrolysates can be used in serum-free formulations for insect cells if extreme safety considerations discourage the use of meat hydrolysates. Meat peptone has been used as a cheap source of amino acids and biomass precursors to reduce the cost of a hybridoma perfusion culture by 80% (23). Hydrolysate amino acids are unlikely to have a similar effect in insect cell culture, however, because of the high amino acid content of insect basal media. Hydrolysates may have a nutritional role if basal media with lower amino acid content or no amino acids are used. Conversely, hydrolysates can perform many serum functions, as shown by the use of yeastolate, which facilitates the purification of recombinant proteins produced in the BEVS system. Integration of an ultrafiltration step in media preparation can eliminate hydrolysate lot-to-lot variability and ensure reproducibly good growth of insect cell lines (21). The practice of insect cell culture with serum-free media described in the following sections is based on both our own experience as well as on well-established procedures previously described (24–26).

## 2. Materials

### 2.1. Material for Monolayer Culture

1. Plastic T-flasks. We prefer the Nunc EasYFlasks™ line from Nalge Nunc International (Rochester, NY, [www.nuncbrand.com](http://www.nuncbrand.com)) available from all major labware distributors worldwide (Fisher Scientific, VWR, Sigma-Aldrich [<http://www.sigma-aldrich.com>], Cole Parmer, MP Biochemicals [<http://www.mpbio.com>]).

www.mpbio.com], and so on). Sizes: 25, 75, and 150 cm<sup>2</sup> (Nunc, cat. nos. 156340, 156472, and 159920, respectively).

2. Sterile plastic cell scrapers (Nunc, cat. nos. 179693 or 179707)
3. Sterile plastic serological pipets of 1–10 mL (Nunc, cat. nos. 159609, 159617, 159625, 159633).

## 2.2. Equipment for Shaker, Spinner, or Bioreactor Culture

1. Orbital shaker with clamps suitable for 125- to 500-mL Erlenmeyer flasks (New Brunswick Scientific, Edison, NJ, <http://www.nbsc.com>; model C24).
2. Disposable plastic 125-mL Erlenmeyer flasks from Corning (Acton, MA, <http://www.corning.com/lifesciences/>; cat. no. 431143) (*see Note 1*).
3. Spinners (100 mL up to 3 L; Bellco, Vineland, NJ, <http://www.bellcoglass.com>; cat. no. 1965 series).
4. Bioreactor (2–7 L; e.g., New Brunswick Scientific, Celligen™).

## 2.3. Cryopreservation of Serum-Free Cultures

1. Ultra-low temperature automated freezer (New Brunswick Scientific, model no. U570).
2. Manual freezer tray.
3. Cell culture grade DMSO (>99.7%, liquid, sterile-filtered, cell culture tested; Sigma-Aldrich, cat. no. D2650).
4. Cell cryopreservation vials (Corning, cat. no. 430656).
5. Fresh complete serum-free medium of choice (*see Subheading 2.4.*).
6. Cell-free conditioned serum-free medium (conditioned medium obtained after a 4-d cultivation of the cell line for whose freezing it is used).

## 2.4. Media and Solutions

Serum-free medium of choice, either complete (no supplementation needed) or basal with supplements (e.g., protein hydrolysates and Pluronic F-68–lipid mixture).

### 2.4.1. Complete Medium

1. Sf-900 II insect cell culture SFM (GIBCO Life Technologies, Invitrogen Corp., cat. no. 10902).
2. Express Five SFM (GIBCO Life Technologies, Invitrogen Corp., cat. no. 10486-025).
3. Insect-XPRESS (Cambrex, cat. no. 12-730Q).
4. EX-Cell 405 (JRH Biosciences, cat. nos. 14405 or 24405).
5. EX-Cell 420 (JRH Biosciences, cat. no. 24420).
6. HyQ SFX-Insect (Hyclone, cat. no. SH30278).
7. TiterHigh™ Sf Insect Medium (Sigma-Aldrich, cat. no. I5408).

#### 2.4.2. Basal Medium

1. IPL-41 Insect medium (1X, liquid, insect cell culture tested; GIBCO Life Technologies, Invitrogen Corp., cat. no. 11405-081).

#### 2.4.3. Supplements to Basal Medium

1. Pluronic F-68 solution (10% solution, sterile-filtered, insect cell culture tested; Sigma-Aldrich, cat. no. P5556 or GIBCO Life Technologies, Invitrogen Corp., cat. no. 24040-032).
2. Lipid mixture (1000X, liquid, sterile-filtered, insect cell culture tested; Sigma-Aldrich, cat. no. L5146).
3. Yeast extract ultrafiltrate (yeastolate) (50X, insect cell culture tested; Sigma-Aldrich, cat. no. Y4375).
4. Primatone RL (MP Biochemicals, formerly ICN Biochemicals, cat. no. 191520).

#### 2.4.4. Cell Viability Determination

1. Trypan blue stain (GIBCO Life Technologies, Invitrogen Corp., cat. no. 15250-061).

### 3. Methods

Most lepidopteran insect cells tend to form clumps and lose viability in the first few passages when they are transferred abruptly to a less hospitable environment than they are adapted to (e.g., directly to suspension from adherent or monolayer culture; to extremes in pH, temperature, or osmolality; to serum-free from serum-supplemented medium). It is a good practice for the cells to be adapted to suspension culture before attempting to grow them in serum-free medium. The absence of a variety of serum-associated proteins and growth factors in the serum-free medium renders the cells more sensitive to poor water quality, bioburden (e.g., endotoxin), or other impurities (e.g., from poorly cleaned vessels). Therefore, the adherence to rigorous cleaning and aseptic practice is an absolute must in serum-free insect cell culture. In addition, this increased sensitivity of the cells in serum-free culture implies the adaptation of classic cell culturing techniques to milder practices, e.g., reduced use (or total elimination) of antibiotics, reduced use (or total avoidance) of trypsinization for dislodging adherent cells from culture vessels, and consistent cryopreservation (freezing) and recovery (thawing) procedures. Cells propagated in serum-free media are also more fragile than cells grown in serum-containing media. Therefore, upon subculturing, insect cells grown in serum-free media must be separated by low-speed centrifugation. In addition, dislodging of adherent cells from culture vessels should be done with mild tapping or shaking or with a gentle stream of medium rather than using trypsin or collagenase.

### 3.1. Routine Monolayer Culture

This procedure is applicable to most lepidopteran insect cell lines in plastic T-flasks or dishes (*see also* Chapter 9):

1. Using a 10-mL pipet, aspirate medium and floating cells from a confluent monolayer, discard and again add about 4 mL of fresh complete medium to a 25-cm<sup>2</sup> T-flask (12 mL to a 75-cm<sup>2</sup> T-flask or 25 mL to a 150-cm<sup>2</sup> T-flask).
2. Resuspend cells by pipetting the medium across the monolayer (sloughing) using a Pasteur pipet. In case of stronger attachment use a gentle but sharp hand motion (wrist-snap shaking motion).
3. Monitor the cell monolayer under an inverted microscope to make certain that complete detachment of the cells from the substratum surface has taken place.
4. Perform viable cell count using Trypan blue exclusion procedure.
5. Inoculate cells in T-flasks at  $2\text{--}5 \times 10^5$  cells/mL (or  $0.8\text{--}2 \times 10^6$  cells/25-cm<sup>2</sup> T-flask).
6. Put T-flasks in an incubator ( $27 \pm 0.5^\circ\text{C}$ ).
7. On day 3 postplanting aspirate spent medium from one side of the monolayer and refeed the culture with fresh serum-free medium gently introduced to the side of the T-flask.
8. Subculture the T-flasks when the monolayer reaches 90–100% confluency. Exponential growth can be maintained by splitting cells at a 1:5 dilution.
9. After >30 passages (2–3 mo in culture) and after increased doubling times (>28 h) have been reached, insect cells lose their viability and it is time to thaw new cell cryovials to initiate new monolayer cultures.

### 3.2. Adaptation to Suspension Culture

The individual details for adaptation of insect cell lines from adherent to suspension culture should be determined empirically. Cell viability should be routinely monitored at each passage (subculturing) and, as a rule, it should be greater than 90%. Most insect cell lines do not adhere to monolayer substrates (T-flasks, dishes) as strongly as anchorage-dependent mammalian cell lines. As previously stated, gentle mechanical force can usually dislodge the adherent cells. Even in the case of Tn-5 cells, which have a higher tendency toward adhering to substratum (in monolayer culture) and will form clumps when put directly in suspension, mild techniques, such as addition of heparin or dextran sulfate (molecular weight [MW] ~approx 12,500 [27]) can contribute to obtaining single-cell suspensions. This may not be necessary in case an already suspension-adapted cell line is obtained from a commercial supplier (e.g., the cells purchased from Invitrogen have been adapted to grow individually in suspension). Thus, monolayer cultures of all types of lepidopteran cells can be used to initiate suspension cultures. All addition and removal of medium should

be done in a biological safety cabinet by using aseptic conditions and scrupulously sterile equipment.

1. Use 6–8 confluent 25-cm<sup>2</sup> T-flask to start a 30-mL suspension culture (6–10 75-cm<sup>2</sup> T-flasks to start a 100-mL suspension).
2. Dissociate cells from the bottom of the flasks with a gentle stream of medium or by sharply rapping the flask against the palm of the hand (wrist-snap shaking motion).
3. Pool cell suspensions from the various T-flasks into the appropriate vessel (Erlenmeyer shake flask or spinner flask) and perform a viable cell count (trypan blue exclusion) (*see* Chapter 1).
4. Add fresh serum-free medium to the cell suspension to reach  $2\text{--}5 \times 10^5$  cells/mL. If the cells have been obtained from a monolayer culture in serum-containing medium, then they may initially form clumps upon transfer to serum-free medium. In such a case, these aggregates can be disrupted by pipetting into and out of a Pasteur pipet. Alternatively, single-cell suspensions can be selected during subculture by allowing the bigger clumps to settle at the bottom of the shake flask or spinner flask and using only the single cells or smaller clumps remaining in suspension (typically within the upper one-third of the suspension) for seeding the next subculture.
5. Incubate shake flasks on the shaker at 90–100 rpm and spinners at a stirring rate of 40–60 rpm in an incubator (or constant-temperature room) at  $27 \pm 0.5^\circ\text{C}$ .
6. Subculture the cells into fresh medium when the viable cell count reaches  $1\text{--}2 \times 10^6$  cells/mL and increase the stirring or shaking speed by increments of 5 rpm as long as viability is not compromised. If it falls to less than 80%, then a decrease of agitation is in order until growth continues and viability exceeds 80%.
7. Repeat **step 6** until a constant agitation speed of 90 rpm for spinner flasks or 130 rpm for shake flasks is reached and a consistent viability above 90% is maintained. Further subculturing from this point can be done at  $0.3\text{--}1 \times 10^6$  cells/mL (i.e., every 1–3 d). Check cells daily for density and viability.
8. In the case of cell aggregate formation, proceed as in **step 4** (i.e., settling of larger cell clumps, selection of single cells and small aggregates from upper third of suspension, and subsequent subculture).
9. Freeze a sufficient quantity of cells adapted to suspension culture to initiate future suspension cultures (*see* **Subheading 3.6.**).

### **3.3. Adaptation to Serum-Free Media: Direct Approach**

The main advantage of the direct method of adaptation to serum-free culture is saving time. Most lepidopteran cell lines can be adapted directly to commercial or to low-cost homemade serum-free media within a span of four to eight passages. If viabilities decrease to <60% or if the cultures are propagating with cell doubling times >40 h for more than three to four consecutive passages,

then the gradual adaptation (weaning) methodology is recommended (*see Subheading 3.4.*).

1. Transfer cells grown in serum-containing conventional medium directly into prewarmed serum-free medium ( $27 \pm 0.5$  °C) at a viable density of  $0.5\text{--}1 \times 10^6$  cells/mL.
2. When the viable cell density reaches  $1\text{--}3 \times 10^6$  cells/mL, subculture the cells to a density of  $0.5 \times 10^6$  cells/mL (within 4–7 d).
3. Subculture the cells in fresh serum-free medium once or twice a week, i.e., when the viable cell density reaches  $2\text{--}5 \times 10^6$  cells/mL and the viability is at least 80%. Both the maximum cell density and the cell doubling time should be comparable to that in serum-containing medium.

### **3.4. Adaptation to Serum-Free Media: Gradual Approach (Weaning)**

1. Subculture insect cells growing exponentially in serum-containing medium into a 1:1 ratio of serum-free and serum-supplemented medium with the viable cell density between  $0.5$  and  $1.0 \times 10^6$  cells/mL.
2. Incubate the cultures until the viable cell density doubles and then subculture cells by mixing equal volumes of the cell suspension (conditioned medium) and fresh serum-free medium (1:1).
3. Continue to subculture the cells in this manner until the serum concentration is reduced below 0.05% with the cell viability remaining >85%. The cell doubling time should be comparable to that in serum-containing medium.
4. A sufficient quantity of the adapted cells should be cryopreserved to initiate future suspension cultures (*see Subheading 3.7.*).

### **3.5. Shake-Flask Culture**

By far the easiest manner to grow lepidopteran insect cells in suspension is to use Erlenmeyer shaker flask culture. These cultures commonly use either 100-mL (working volume of 30–40 mL) or 250-mL (working volume of 80–100 mL) flasks. Aeration is accomplished by loosening the cap approximately 1/4 turn (alternatively, an Erlenmeyer flask covered with aluminium foil can be used). This approach eliminates oxygen limitations to the cells, thereby allowing cells to grow at their maximum rate. A generic protocol is given here that should work for any lepidopteran insect cell line.

1. Inoculate the desired number of 250-mL Erlenmeyer shake-flasks with 80–100 mL of complete serum-free medium containing  $3 \times 10^5$  viable cells/mL at a viability of at least 90%. Alternatively, the desired number of 125-mL shake-flasks can be inoculated with 30–40 mL of serum-free medium containing  $3 \times 10^5$  viable cells/mL.

2. Set the orbital shaker to an agitation speed of 125–140 rpm, depending on the characteristics of the cell line and the medium. If the cells have been newly adapted to suspension and serum-free medium, a lower shaking speed is recommended initially. Maintain the shaker assembly in an environment of  $27 \pm 0.5^\circ\text{C}$ .
3. When the culture reaches  $1\text{--}3 \times 10^6$  viable cells/mL, split the shake-flask contents to subculture the cells to about  $3 \times 10^5$  viable cells/mL into similar size shake flasks with fresh serum-free medium. The subculturing should be done when the cells are in midexponential growth and at maximum (>90%) viability.
4. Every 3 wk, cultures may be gently centrifuged (100–200g for 5 min) and the cell pellet resuspended into fresh complete serum-free medium to reduce cell debris and toxic metabolite accumulation.

### 3.6. Spinner Culture

Although spinner culture is practiced less widely than shake-flask culture, it has the advantage of simulating an environment closer to that of a stirred bioreactor and, hence, this type of suspension culture may be useful in the case of scale-up. A spinner flask with a vertical impeller is preferred over that with a hanging stir-bar, because it supplies better aeration (*see Note 2*). Before starting, it is important to recalibrate the working volume of the spinner flasks using a graduated cylinder or volumetric flask as a reference. This calibration should be done with the impeller removed from the vessel. Two protocols are given for spinner culture, the first for *S. frugiperda* cells (Sf-9, Sf-21) and the second for *T. ni* cells (Tn-5).

#### 3.6.1. Spinner Culture for *S. frugiperda* Cells

1. Establish an adequate supply of exponential phase monolayer culture cells to start a spinner of desired size (typically, 100- or 250-mL) with  $10^6$  viable cells/mL. Typically four to six confluent 75-cm<sup>2</sup> T-flasks are required to initiate a 100-mL culture (four to five flasks for the spinner and one as a backup).
2. Remove cells from flasks (dislodging as described in **Subheadings 3.1.** and **3.2.**), pool them together, count cells and verify that the viability is at least 90%.
3. Seed one or more clean, sterile spinners with enough cells to reach a maximum density of  $10^6$  viable cells/mL (a minimum of  $3 \times 10^5$  cells/mL).
4. Incubate the spinner(s) at  $27 \pm 0.5^\circ\text{C}$  with constant stirring at 75–90 rpm. The top of the impeller should be slightly above the medium surface, to provide additional aeration. Loosening the side arm caps on the vessels ( $1/4$  turn) will provide equilibration with the atmosphere.
5. When the cells reach a density of  $2\text{--}3 \times 10^6$  viable cells/mL, add sufficient quantity of fresh serum-free medium to the spinner(s) to bring the cell density back to  $10^6$  cells/mL. This should be done every 1–3 d.
6. Alternatively, you may reseed new, sterile, and clean spinners (containing fresh serum-free medium) to about  $3 \times 10^5$  cells/mL.
7. Cells should be monitored daily for density and viability.

### 3.6.2. Spinner Culture For *T. ni* (Tn-5) Cells

The Tn-5 insect cell line is more adherent than the Sf-9 and Sf-21 cells. Serum-free medium supplemented with heparin (or dextran sulfate) is used to reduce cell clumping in suspension culture. The following protocol is recommended for Tn-5 cells from monolayer cultures.

1. Start with four confluent 75-cm<sup>2</sup> T-flasks of monolayer adherent Tn-5 cells, dislodge the cells by sloughing, and inoculate a 100-mL spinner to a volume of 40–50 mL and a cell density of  $5\text{--}7 \times 10^5$  cells/mL. Add fresh serum-free medium to adjust the volume or cell density and add 10 U heparin/mL culture.
2. Incubate the spinner at  $27 \pm 0.5^\circ\text{C}$  with constant stirring at 75–90 rpm. The top of the impeller should be slightly above the medium surface, to provide additional aeration. Loosening the side arm caps on the vessels ( $1/4$  turn) will provide equilibration with the atmosphere.
3. After 1 d perform a viable cell count and determine cell viability. The latter should be  $>90\%$ . If the cell density has not reached  $2 \times 10^6$  cells/mL, then incubate for additional time.
4. Upon attaining a cell density of  $2 \times 10^6$  cells/mL or higher, passage the cells with heparin into a second 100-mL spinner with fresh serum-free medium to a cell density of  $10^6$  cells/mL.
5. Passage the spinner flasks when the cell density of  $2 \times 10^6$  cells/mL or higher is reached. The cells must be monitored in terms of density and viability every 24 h. The heparin can be eventually weaned, if cell clumping decreases in successive passages. The cells will be considered as fully adapted to spinner culture when a high viability (e.g.,  $>95\%$ ) and a doubling time of 18–24 h are maintained.
6. Pool the cells from the two 100-mL spinners into one 500-mL spinner and add 150 mL of fresh serum-free medium (supplemented with heparin if clumping persists). The final cell density should not be below  $8 \times 10^5$  cells/mL.
7. Continue to grow and passage the cells in spinners of 500 mL (or 1000 mL), following the procedure of **step 4**, until the viability is  $>95\%$  and the cells' doubling time stays between 18 and 24 h. At this point the gradual removal of heparin (if it is still being used) can be accomplished. Culture of this volume and physiological characteristics (in mid-exponential phase) can be used for maintenance culture or for inoculating a vessel on a higher scale, e.g., a Celligen bioreactor.

## 3.7. Cryopreservation

### 3.7.1. Freezing

In the protocol detailed next, cells are cryopreserved by freezing concentrated culture aliquots in a cryopreservation medium consisting of the serum-free medium in which the cells have been grown plus a solution of dimethyl sulfoxide (DMSO).

1. Cells for cryopreservation are grown in shaker or spinner culture and are harvested in mid-exponential phase at a viability exceeding 90%.

2. Prepare freezing medium, consisting of 45% fresh cold serum-free medium, 45% spent serum-free medium, and 10% DMSO. Chill freezing medium and hold at 4°C.
3. Centrifuge the cells at 100–200g for 5 min. Discard the supernatant and resuspend the cell pellet in an appropriate volume of freezing medium to yield a suspension of  $1\text{--}2 \times 10^6$  cells/mL.
4. Rapidly transfer suitable volume of the homogeneous cell suspension to corresponding cryovials (e.g., 4- or 5-mL cryovials).
5. Place the cryovials at 4°C for 30 min, at –20°C for 3–4 h, and at –70°C for 16–24 h. Just before transferring the vials from 4°C to freezing gently mix the contents to ensure homogeneity. Following the time at –70°C the vials can be transferred to liquid nitrogen, where they can be preserved indefinitely.
6. If an automated freezer apparatus is available, then a freezing rate of 1°C/min should be used.

An alternative procedure is to use the serum-free medium for cryopreservation in conjunction with an appropriate specialized cryopreservation solution. For example, Insect-XPRESS 1X liquid can be mixed in a 50:50 (v/v) proportion with Cryoprotective Medium (Cambrex, cat. no. 12-132) to provide the appropriate environment for the cells to be preserved in cryovials.

### 3.7.2. Thawing

The recovery (thawing) of cryopreserved cells can be done as follows:

1. Rapidly thaw a vial of frozen cells in a 37°C water bath.
2. Transfer the cells aseptically to a centrifuge tube containing 10 mL cold serum-free medium.
3. Using low-speed centrifugation (200g for 5 min) recover the cell pellet and resuspend in 5 mL fresh serum-free medium.
4. Determine cell density and viability and transfer the cells to a sterile shake flask at a seeding density of  $2\text{--}4 \times 10^5$  cells/mL.
5. Follow **steps 1–7** of shake-flask culture (**Subheading 3.5.**).

### 3.8. Quantitative Assessment of Growth and Infection Phase

See Chapter 1 for details. Briefly, growth is evaluated in terms of population doubling time, specific growth rate ( $\mu$ ), growth extent (maximal cell density or cell density at infection), length of the stationary/decline phase and viability; infection at a given multiplicity of infection (MOI) is assessed by the evolution of the postinfection cell density and viability and, most importantly, by the time-course of the viral or recombinant protein titer. Cell density at infection, MOI and time of harvesting are parameters subject to optimization for different recombinant products. Starting from seeding densities of  $2\text{--}4 \times 10^5$  cells/mL, maximal cell densities between 5

and  $8 \times 10^6$  cells/mL for Sf-9 and Tn-5 cells are typically obtained on day 5–7 postinoculation in batch shake-flask culture and in suspension bioreactors, depending on the serum-free formulation and the passage number of the inoculum. These values can easily increase by 100% in cases of fed-batch culture, especially when medium replacement or specific nutrient supplementation is practiced (2).

### **3.9. Recommended Best Practices in Handling and Storing Serum-Free Media**

The serum-free medium should be free-flowing, clear and free of precipitates or flocculent matter. Deteriorated media are altered in color (fresh media are typically light yellow, but some variability may be acceptable in some media, *see Note 2*), have a pH outside the range 6.0 to 6.4 and their performance is suboptimal. Upon receipt, serum-free media should be stored at 2–8°C and protected from light and from excessive exposure to the ambient air. This is important, because several of their ingredients, such as group B vitamins and lipids, tend to be photodegraded or oxidized. Excessive exposure to air can be avoided by aseptically overlaying the opened bottle of medium with nitrogen gas and capping it tightly after each use. In any case, opened bottles of serum-free media do run the risk of contamination if they are stored for periods beyond a few weeks.

Although homemade serum-free media are formulated from a basal medium, e.g., IPL-41, by supplementing with additives (e.g., surfactants and shear-protective solutions such as a Pluronic F-68–lipid mixture) and serum replacements (such as protein hydrolysates), the vast majority of commercially available media are complete and ready to use (unless otherwise explicitly indicated) and, therefore, they do not need to be supplemented with additives. Supplementation of ready to use commercial serum-free media with Pluronic F-68 may lead to an excessive content of this ingredient, already present in the majority of commercial media, and cause toxicity to the cells (this would take a high concentration; using purified Pluronic F-68 should avoid this at any rate). With respect to serum-free media explicitly designated as devoid of L-glutamine, this amino acid should be supplemented in accordance to the protocol of the relevant application (bioprocess of interest), typically at a final concentration of 15–17 mM.

It is recommended to maintain separate media bottles for the cultivation of insect cells and for work with baculovirus (recombinant or wild type). Virus particles can survive in media stored in the refrigerator (2–8°C) and subsequently contaminate cell cultures if medium from a virus-contaminated bottle is used for passing cells in T-flasks or shake-flasks.

All serum-free media must be allowed to equilibrate to room temperature before they are used for culturing activities.

### **3.10. Insect Cell Infection in Serum-Free Culture**

Failure or suboptimal performance upon infection of insect cells in serum-free culture is most often the result of nutrient limitations. Accumulation of inhibitory by-products, e.g., ammonium ion or lactate, could also account for this behavior. Either of these phenomena can be overcome by resuspending the infected insect cells in fresh medium following centrifugation of the cell suspension.

### **3.11. Development of New, Low-Cost, In-House Serum-Free Media**

As stated in the introduction, the single development that has offered new “traction” in the never-ending quest for superior serum-free media is the replacement of serum with supplements, mostly protein hydrolysates, that offer not only a nutritional reinforcement as sources of amino acids but also provide specific biological activity (growth factors, antioxidants, antiapoptotic factors, and so on). Their origin (like that of any other medium component) is increasingly scrutinized because of regulatory concerns, with a clear trend toward hydrolysates of nonanimal origin (i.e., primarily microbial or plant peptones). The separation of low MW fractions of hydrolysates with specific activities by chromatographic methods can offer better chemical definition of culture media and improve the downstream purification of recombinant proteins (28,29). The lessons gained from mammalian cell culture, in relation to the biological effects of specific oligopeptides, can be adapted and extended to insect cell culture either by direct testing on insect cell lines or by analysis of a variety of hydrolysates. A good starting point should be the analysis of yeastolate ultrafiltrate, which is a striking example of a multifunctional hydrolysate, with a central role in the culture of a wide variety of insect cell lines. Yeastolate ultrafiltrate is a major component of serum-free media (13,19,22,30) and an efficient supplement for high-density fed-batch culture of insect cells (31,32) and for the restoration of protein productivity by the BEVS (33–35). The yeastolate components responsible for boosting cell growth and restoring protein production have not yet been identified (36,37), but recent progress indicates that these phenomena are complex, multifactorial and probably owing to both high- and low-MW components (37).

In the early 1990s a key question regarding insect cell culture was “Is the near future just a period of fine tuning of existing media or will there be another breakthrough in design of cell culture media?” (20). It has not been answered yet, at least not fully. Clearly, there is no major breakthrough at

present, but the rational use of metabolic knowledge and an increased understanding of the specific biological effects of serum substitutes, such as hydrolysates, can lead to one. Medium development goes hand in hand with the development of new cell lines and understanding the cells' metabolism. Novel cell lines for baculovirus infection were reported during the last decade (7,8,38–43), but only a couple of them have found their way into mass production, mainly because of regulatory hurdles. As for our knowledge of insect cell metabolism, it still comes mainly from the Sf-9 cell line (44–49) with limited information on the Tn-5 cell line (50,51). This knowledge is exploited for empirical (see **Subheading 3.11.1.**) or statistical, primarily factorial (see **Subheading 3.11.2.**) experimental design of new serum-free media. Glucose is widely recognized as the most important carbon source (44,52,53) and there is, at least, one medium containing glucose as the sole carbohydrate (54). Specific glucose consumption rate is, in general, higher for Tn-5 compared to Sf-9 cells (19,50); thus, a high initial concentration is necessary for serum-free media destined for both cell lines. Glutamine, another important metabolite in animal cell culture, has been shown not to be essential for the culture of Sf-9 and Sf-21 cells (55). Tn-5 cells, however, could not grow in glutamine-free medium (55). This point as well as the preferential consumption of asparagine over glutamine by Tn-5 cells and the excessive ammonia production from the latter cell line, are points to be considered for design of media supporting several distinct lepidopteran cell lines. Amino acid utilization rates may be different before and after infection (50,56) and this information is vital for the development of new serum-free formulations or even basal media. Once again, factorial designs (see **Subheading 3.11.2.**) may be useful for the simultaneous optimization of several amino acid levels. Systematic exploitation of data from metabolic studies, use of statistical methods and expansion of protein hydrolysates of nonanimal origin can produce wide range serum-free media, efficient for both growth and infection phases and suitable for downstream processing.

### 3.11.1. Empirical Approach (21,22)

As the name implies, in this approach there are no formalized rules to follow and the procedures do not lend themselves to strict step-by-step protocols. Nonetheless, a typical path toward formulating a new medium can be as follows:

1. Start with a basal medium requiring serum supplementation, such as Grace's (10), TNM-FH (6), or, preferentially, IPL-41 (12), which has a relatively simple formulation and is easy to filter.
2. Retain levels of glucose and of glutamine commensurate with the requirements of the lepidopteran cell line to be cultured.

3. Add the lipid mixture emulsified in Pluronic F-68 (13). Alternatively, if the level of Pluronic F-68 is to be varied, use the lipid mixture (1000X, liquid, sterile-filtered, insect cell culture tested; Sigma-Aldrich, cat. no. L5146) mixed directly with Pluronic F-68 (10% solution, sterile-filtered, insect cell culture tested; Sigma-Aldrich, cat. no. P5556 or GIBCO Life Technologies, Invitrogen Corp., cat. no. 24040-032) or mix the lipid mixture directly in the medium.
4. Replace serum and amino acids with hydrolysates (or, alternatively, with different defined peptides and vitamins).
5. Mix together all components of the serum-free medium formulation under development and then filter them through a 0.2- $\mu$ m filter.
6. Test each component by titrating it individually to determine its optimal level of supplementation. Use growth rate, viability and growth extent (maximal or final cell density) as criteria for the growth phase, and growth extent, viability, and product virus or protein titer as criteria for the infection (production) phase.
7. **Steps 4–6** must be repeated as different components are added, to exclude combinations that have an adverse effect on growth and/or production.

### 3.11.2. Statistical Approach (19)

An emerging line of research and development is the use of factorial experiments for the design of new, rationally designed serum-free media or the screening of supplements. Factorial designs are a unique way to detect interactions between the parameters tested (57), allow the researcher to test many variables at the same time and can greatly reduce the number of experimental runs needed. Thus, their use can result in great time and cost savings. In insect cell culture, a fractional factorial experiment was employed for the screening of several hydrolysates and a subsequent full factorial experiment for the optimization of the selected hydrolysate (yeastolate and Primatone RL) concentration (19). The same basic experimental design can be applied to the development of new media for growth and infection of any lepidopteran cell line. Here is an illustrative sequential list of procedures:

1. Depending on the media components to be screened and subsequently optimized, set up one or more  $2^n$  factorial matrices. For instance, in the case of our screening of seven different hydrolysate supplements for the replacement of serum, to identify the effect of each of them on insect cell growth and any possible interactions between hydrolysates we would need a full  $2^7$  factorial experiment (19). Because to perform  $2^7$  tests was impossible in practice, the estimation of the principal effects and interactions was based statistically (57) on a  $2^{7-4}$  fractional factorial experiment, i.e., on a matrix containing groups of hydrolysate combinations (19). The corresponding matrix can be set up to indicate the absence or presence (or, alternatively, the low or high level) of a component (in this case of a given hydrolysate). In an *ab initio* (from scratch) formulation of a medium or in the case of its optimization for increased performance, two  $2^3$  factorial matrices can

be established, grouping two sets of three components each: the first one (I) testing amino acids, salts and C/energy source (e.g., sugar[s] and glutamine), the second one (II) testing vitamins, hydrolysates, and lipids (e.g., plant-derived fatty acids to replace the methyl ester fatty acids from cod liver oil, to arrive at an animal component-free formulation).

2. Carry out the tests dictated by the above factorial combinations, using the growth extent as the response variable for the growth phase, and the product virus or protein titer as the response variable for the infection (production) phase. The overall growth extent can also be assessed by the integrated surface area below the cell growth curve, in terms of cell-days.
3. Do a statistical analysis of the quantitative responses obtained. For this, estimate the main effects by equations summing up the responses of the different component combinations (57). The weighted overall effect of an individual component A is the difference between the fraction (mean)  $y_{A+}$  of the responses from combinations containing component A and the mean  $y_{A-}$  of the responses from combinations lacking component A. For instance, in the screening of the seven different hydrolysate supplements (indicated as A, B, C, D, E, F, G) for the formulation of our serum-free medium YPR using a  $2^{7-4}$  fractional factorial experiment (19), the combinations tested were the following  $2^3$ : D + E + F, A + F + G, B + E + G, A + B + D, C + D + G, A + C + E, B + C + F, and A + B + C + D + E + F + G. The values of the responses corresponding to each of these combinations could be designated, respectively, as **def**, **afg**, **beg**, **abd**, **cdg**, **ace**, **bcf**, **abcdefg**. Thus, the overall effect for hydrolysate A could be obtained by the following equation (19,57):

$$\text{Effect}_A = y_{A+} - y_{A-} = (\text{abd} + \text{afg} + \text{ace} + \text{abcdefg})/4 - (\text{def} + \text{beg} + \text{cdg} + \text{bcf})/4 \quad (1)$$

As an alternative to this type of calculation and rating of overall effects, a statistical analysis program, such as Design-Expert® (software from Stat-Ease, Inc., <http://www.stat-ease.com>) can be used.

4. Retain those components (or levels of components) whose effects are most significant (numerically higher and positive) and eliminate those components whose effects are negative or negligible. In the process of elimination, do not use only these numerical data but look for qualitative trends (e.g., in the case of insect cell growth, the prolonged stationary/decline phase at a reasonably high viability).
5. Perform a complete factorial experiment using the components that have been identified and retained from the previous step. For instance, if two (2) components have been retained as significant, then set up a  $2^2$  factorial matrix testing their levels and interactions. In the case of the two separate  $2^3$  factorial matrices - grouping two sets of three components each as suggested in **step 1** for *ab initio* medium formulation, set up a  $2^2$  factorial matrix testing the predicted supplement levels from the two grouped combinations (I and II).
6. Carry out the tests suggested by the combinations of **step 5**.
7. Do a statistical analysis of the quantitative responses obtained as indicated in **step 3**.
8. Test the optimized medium under different conditions (e.g., in bioreactors).

### 3.11.3. Genetic Algorithm Approach (58,59)

A novel research tool that has been recently proposed for medium design and optimization in insect cell culture is the use of genetic or evolutionary algorithms (58). This strategy is designed to assess the effects of combined multiple parameters on a given outcome and, hence, can be adapted to many complex multiparameter optimization problems (59,60). The basic idea of the genetic algorithm is based on Darwinian selection, i.e., in a system of *several* inputs (parameters, such as media components) the tendency after some rounds of mutation and selection (or mating among the fittest individuals) is toward *one* output (fitness, such as an optimal growth-supporting combination). The input parameters can be seen as genes with different values (alleles). A defined combination of values makes up a genome (parameter set) corresponding to an individual experimental set up, whose outcome is the fitness. Different individuals are grouped into batches (generations) that are allowed to mate. Based on the fitness of the individuals emerging after mating (different parameter combination) leads in the next generation to a result of higher fitness (the genomes of the best individuals are recombined in a new, fitter set of individuals). This whole process can now be formalized with the use of appropriate software packages that allow the researcher to execute the genetic algorithm calculations. The use of genetic algorithms in insect cell media optimization makes it possible to test a whole range of concentrations and a high number of components with either a known or an unknown effect, to test in a relatively limited set of experiments, without the need for educated guesses on concentrations or possible interactions between components (58,60). Furthermore, genetic algorithms can help optimize not only a medium but also a set of conditions (e.g., feeding schedules for a fed-batch culture) using a small number of parallel experiments in only a few generations (testing of previously selected conditions), with a high probability that the result obtained will be close to the optimum, even if it is not the absolute best combination (58,60). Although it is difficult to condense this sophisticated approach into a step-by-step list of procedures, the methodology can be summarized as follows according to ref. 58:

1. Generate a large number (a population) of experimental conditions (individuals) within a parameter space. Parameters are the different nutrients and the parameter space consists of the concentrations that these nutrients are allowed to have. As an illustration, 11 parameters including hydrolysates, amino acids, glucose, lipid mix, and so on, were selected and 31 levels were set in the application seeking to optimize a feed medium for insect cell fed batch culture (58). The initial population (generation 1) is to be generated at random.
2. Encode each component concentration (parameter) by a 5-bit “gene” resulting, in the illustrative case of (58), in a total string length of 55 bits ( $5 \times 11$ ). A string represents an experimental condition (individual).

3. Fix the population of experimental conditions (individuals) to 20 as per (58), given that this value is large enough to be able to reach all points in the parameter space with 99.8% probability (60).
4. After the first set of experiments is carried out and assessed, select the better performing individuals based on the determined maximum viable cell density (growth extent) and the growth rate. Leave the best performing individuals unchanged and allow the others to mate, in performance order, with different individuals. This mating consists of a crossover between the two individuals. Random mutations (variations in conditions) are introduced into this evolutionary process.
5. Set the probability for crossover (mating) to occur at 95% and the mutation rate at 1% per bit according to ref. 60.
6. Always include the best individual in the next generation. Record the outcome of all experiments (combinations), seeking to optimize maximum cell density (growth extent) while retaining a normal or improved growth rate. Include in the optimization the logarithm of the maximum viable cell density with a weight of 1 and the growth rate with a weight of 0.25 (58). The genetic algorithm calculations can be executed by using the software package Galop generated at the Institute of Biotechnology at Jülich, Germany (58).
7. After selection, mating and mutations, a new generation (population) is ready for testing. Produce (with appropriate initialization of parameter values) new generations, until a suitable result is attained or no significant improvement is found anymore (58).

#### 4. Notes

1. The orbital shaker bearing the shake flasks should be housed in a  $27 \pm 0.5^\circ\text{C}$  nonhumidified, nongas regulated environment (e.g., an incubator of appropriate size for the orbital shaker).
2. The total culture volume in a spinner should not exceed one-half the indicated (graduated) volume of the spinner, to ensure proper aeration. For instance, a 500-mL spinner should contain no more than 250 mL of cell culture. A minimum volume is also important, so the impeller stays submerged (typically 30 mL for a 100-mL capacity spinner, 80 mL for a 250-mL spinner, and 200 mL for a 500-mL spinner). Always in the interest of adequate aeration (crucial for insect cell culture), the spinners should be checked for smooth rotary motion of the impeller (avoid jerky or jumping motion).
3. Certain commercial serum-free media contain ingredients that contribute to the medium's hue, but the latter may vary between lots because of the variability in carbohydrate processing of the raw material. In this case the difference in coloration between two lots of medium does not affect medium performance.

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

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### Summary

The various methods for maintaining (a.k.a., 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 (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 260 distinct cell lines have been reported from more than 50 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 who 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 next 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 (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 3 yr ago ([1](#)) because few changes have been made in procedures since that publication. In addition to the procedures outlined next, novices to cell culture should also refer to some general cell culture publications ([2,3](#)). Although 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<sub>2</sub> incubators) and, as poikiotherms (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, although the author has outlined subculture procedures next for enzymatic detachment of cells (e.g., trypsinization) that is commonly used with vertebrate cells, 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 analysis is used, which is 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 the author has provided a method for screening for these agents 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 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- $\mu$ L pipet tips.
6. Trypsin diluent: 800 mg NaCl, 20 mg  $\text{KH}_2\text{PO}_4$ , 20 mg KCl, 150 mg  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 23 mg  $\text{Na}_2\text{EDTA}$  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- $\mu$ m 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. Trypan blue solution (0.4%) (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 (pH 7.5), 25 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 15 mg MTT tetrazolium, 15 mg NADP, 10 mg phenazine methosulfate, 30 mL  $\text{H}_2\text{O}$  (*see Note 3*).
5. Isocitrate dehydrogenase substrate solution (ICD): 2.18 g Tris (pH 7.5), 100 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 50 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 50 mg DL- $\text{Na}_2$ -isocitrate, 15 mg MTT tetrazolium, 15 mg NADP, 10 mg phenazine methosulfate, 30 mL  $\text{H}_2\text{O}$  (*see Note 3*).
6. Phosphoglucose isomerase substrate solution (PCI): 2.18 g Tris (pH 8.0), 76 mg  $\text{Na}_2\text{EDTA}$ , 120 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 210 mg a-d-glucose-1-phosphate, 6 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL  $\text{H}_2\text{O}$  (*see Note 3*).
7. Phosphoglucomutase substrate solution (PGM): 2.18 g Tris (pH 8.0), 76 mg  $\text{Na}_2\text{EDTA}$ , 120 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mg fructose-6-phosphate, 25 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL  $\text{H}_2\text{O}$  (*see Note 3*).
8. Extraction buffer: 10% (v/v) Triton X-100 in 0.6 M Tris-EDTA buffer (pH 7.5) in demineralized water.
9. Stabilization buffer: a proprietary material containing sorbitol and Tris buffer (*see Note 3*).
10. Glycerol.
11. Dimethylsulfoxide (DMSO; sterilized and cell culture tested).

12. Glass freezing ampoules (capped with aluminum foil and heat-sterilized or autoclaved).
13. Long Pasteur pipets, cotton plugged and autoclaved.
14. Aluminum canes for ampoule storage.
15. 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. Electrophoresis temperature-controlled cover and base.
9. Liquid nitrogen Dewar.
10. Ampoule-sealing apparatus or gas/air torch with glass rod.
11. 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 approx 2 mL 70% ethanol (keep a 100- to 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 ultraviolet 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  $\times 10$  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 owing to a cloudy appearance to the medium suggest a bacterial contamination. Such cultures should be autoclaved and discarded.

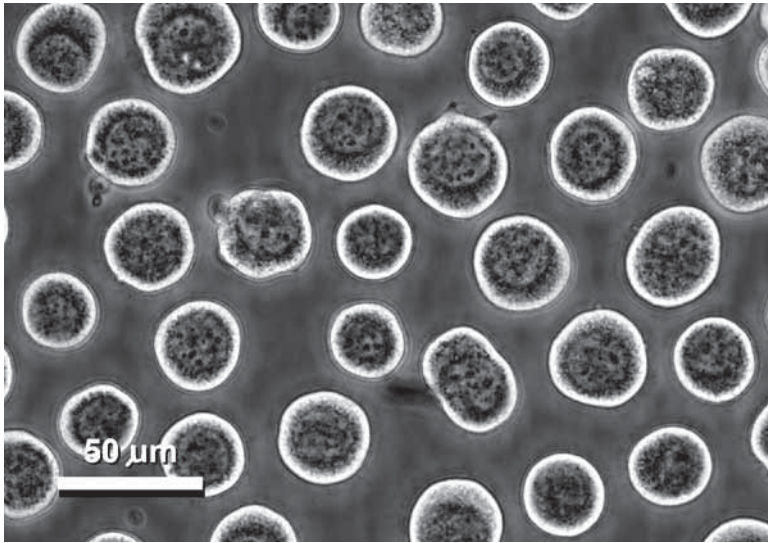


Fig. 1. *Lymantria dispar* fat body cell line, IPLB-LdFB: an example of an insect cell line that grows in suspension. Phase contrast microscopy.

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 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 (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<sup>2</sup> 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 brassica* 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<sup>2</sup> 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. Although 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 previously listed.
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. 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<sup>®</sup>) (Tn-5) and IPLB-TN-R<sup>2</sup> (**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.

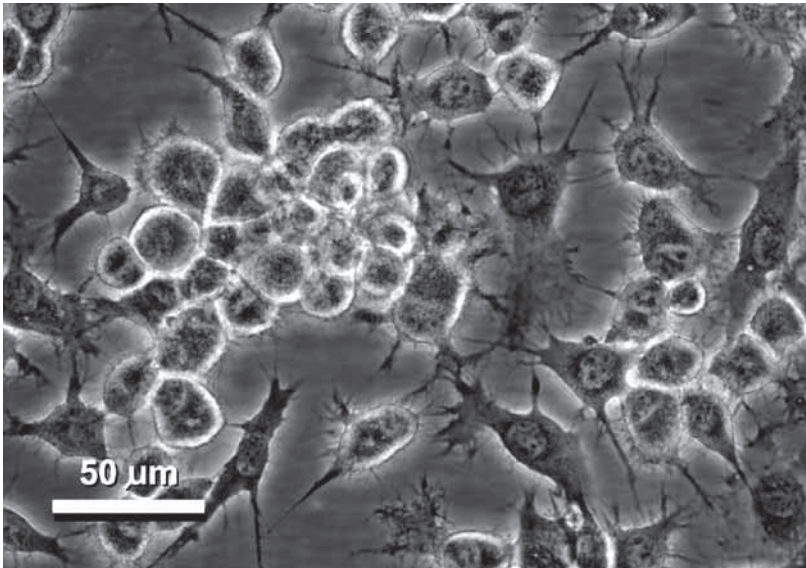


Fig. 2. *Trichoplusia ni* IPLB-TN-R<sup>2</sup> cells: an attached cell line. Phase contrast microscopy.

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<sup>2</sup> 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. Although 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 previously listed 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 previously described.
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.

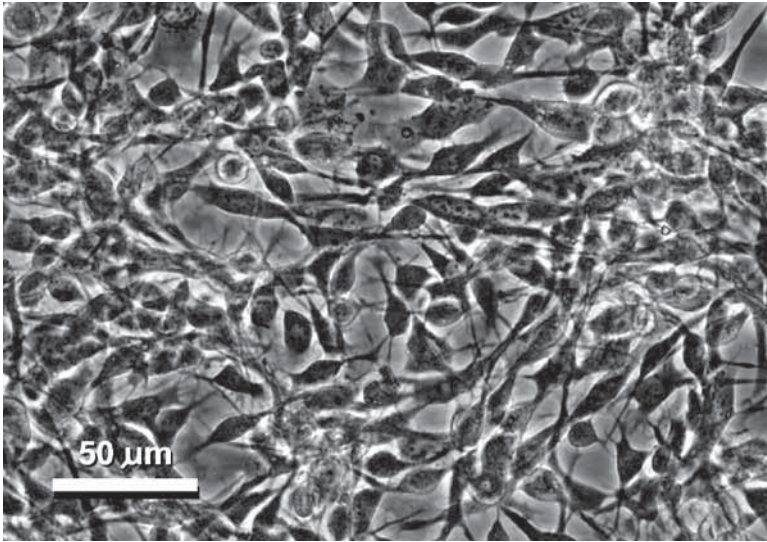


Fig. 3. *Heliiothis virescens* IPLB-HvE1a: firmly attached cells requiring trypsinization. Phase contrast microscopy.

#### 3.1.4. Strongly Attached

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

##### 3.1.4.1. CELL TRYPsinIZATION

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. 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 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 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 min more. 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, tilt the culture again to wet the cell monolayer, lay it flat and wait 5 min more. Repeat this process until the cells are loose.

#### 3.1.4.2. CELL TRANSFER

##### 3.1.4.2.1. Preferred Procedure

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 previously described. 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<sup>2</sup> 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. Although 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. Although some foaming will occur in this process, care should be taken to minimize this because it can result in more damaged cells. Draw in the appropriate amount of the cell suspension (in the previously listed 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 previously listed.

3.1.4.2.2. *Alternate Procedure.* The previously described 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).

1. 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.
2. 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. The author found this is a time consuming step that does not greatly improve the probability of maintaining healthy cultures, but this is largely because the author feels confident in recognizing healthy cells just by examining them in the flask with the inverted microscope. Beginners may want to include this step until they gain confidence in their visual inspection of cells.

### *3.1.5. 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 as a backup. The author typically uses a 17°C incubator for this purpose (other researchers place them in a laboratory drawer, and so on, i.e., at room temperature). Adding some fresh medium to the mature culture will assure that the cells can be recovered for 2 or 3 wk. 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 previously described 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 nonexist-

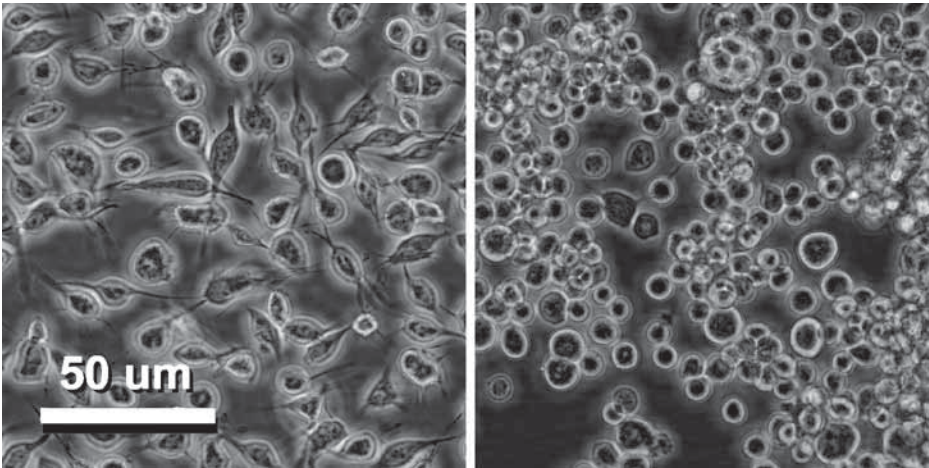


Fig. 4. *Anticarsa gemmatalis* UFL-Ag286 cells. (A) Grown in TC-100 medium, (B) in Ex-Cell 400 serum-free medium.

ent, 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 testing for microbial contamination in most cases. We typically use 100 mL of a 5-L batch of lab-prepared medium and, in my experience, sterility checking of commercially prepared medium has never been 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 because 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.

Mycoplasmas 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 extra-nuclear DNA:

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

Cell line identity is an important issue in cell culture. Nelson-Rees and Daniels (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). The method described here relates to the use of Innovative Chemistry's AuthentiKit™ system (*see Note 3*).

#### 3.2.2.1. ISOZYMES

1. Use cells in exponential growth (typically a 5- or 6-d old culture of cells maintained on a weekly subculture interval).

2. Suspend into the medium using the appropriate method described in **Subheading 3.1**.
3. Transfer cells to a centrifuge tube and pellet at 1000g for 10 min.
4. Rinse the cells by resuspending in PBS.
5. Centrifuge at 1000g 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<sup>2</sup> flask, this is typically between 25 and 100  $\mu$ L).
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 1000g for 10 min.
11. Mix the supernatant with an equal volume of stabilization buffer. This material can be stored at  $-20^{\circ}\text{C}$  for several months. Apply 1  $\mu$ L 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  $4^{\circ}\text{C}$ ). 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 electrophoresed 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  $26\text{--}28^{\circ}\text{C}$  for 20 min (*see Note 7*).
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  $65^{\circ}\text{C}$ ) 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 more than 1 hr.)
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**).

### 3.3. Storage

Keeping a stock of cells in some form of storage is insurance against contamination events, deficient media, or change of cultures over time because of selection of cell types inappropriate to experimental needs (*see Note 8*). As previously mentioned, 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.

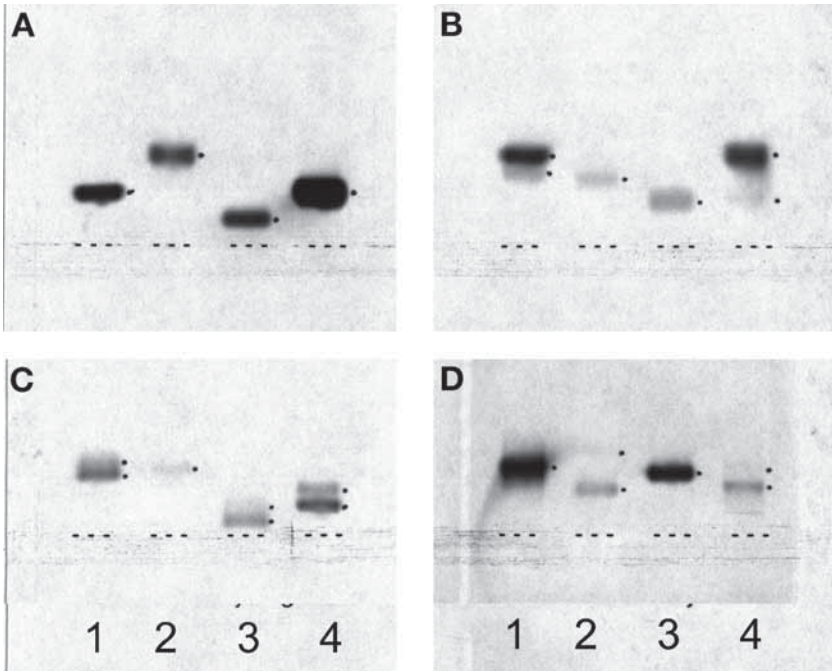


Fig. 5. Isozyme gels. (Lane 1) IPLB-Sf21AE cells; (Lane 2) IPLB-CPB2 cells; (Lane 3) IPLB-DU182E cells; (Lane 4) IPLB-PxE1 cells. (A) Stained for phosphoglucose isomerase (PCI); (B) phosphoglucomutase (PGM); (C) isocitrate dehydrogenase (ICD); and (D) malic enzyme (ME).

### 3.3.1. Low-Temperature

As mentioned in Chapter 7, lower incubation temperatures (16–18°C) can be a useful means of maintaining different cell types in culture. Winstanley and Crook (7) 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 will need to be determined for each cell line of interest but the author found 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<sub>2</sub>) storage (*see Note 9*). Cells stored in LN<sub>2</sub> are very stable, although the freezing and thawing require special

consideration because 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, although 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. The author prefers glycerol because it is a common substance naturally used by some cold-tolerant insects, suggesting it would have fewer adverse effects on the cells but the author has also had some cell lines which 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.

### 3.3.2.1. FREEZING

1. Label the sterilized ampoules with a permanent marker, providing enough information to identify the cells. The author typically uses a brief cell line identity (for example, for the IPLB-Sf21AE cell line, the author would label as SF21) and the passage number.
2. Using the biosafety cabinet/laminar flow hood, suspend a late, exponential phase culture (typically 5 or 6 d old for cultures kept on a weekly subculture interval) by the normal subculture method (**Subheading 3.1.**). The author typically prepares 10 ampoules for freezing from a 75-cm<sup>2</sup> flask (*see Note 10*).
3. Transfer the cells to a 15-mL sterile centrifuge tube and centrifuge at 50–100g for 5–10 min.
4. While the cells are centrifuging, prepare the freezing medium. This is the normal culture medium (*see Note 11*) 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<sup>2</sup> flask).
6. Use the sterile, cotton-plugged long Pasteur pipets to transfer 1 mL to each glass freezing ampoule (*see Note 12*). 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 13*).
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 the ampoules containing the red dye.
10. Place the remaining ampoules on the canes in the freezing apparatus and start the freezing process (*see Note 14*).

11. At the end of the freezing process, transfer the canes to the LN<sub>2</sub> 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.

#### 3.3.2.2. 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, approx 150- to 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* the cautions in **Notes 9** and **13**).
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<sub>2</sub> freezing are prescored for easy breaking. If this is not the case, 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 50–100g for 5–10 min.
8. Discard the supernatant, resuspend the pellet in 4 mL fresh medium, and transfer to a 25-cm<sup>2</sup> tissue culture flask and incubate at 26–28°C.
9. Examine the culture at 1- to 3-d intervals and begin regular subculture procedures (**Subheading 3.1.**) once the culture reaches confluency.

### 3.4. *Baculoviruses*

Hundreds of lepidopteran species have been reported with baculoviruses (**8**), 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 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 their use in continuous cell lines.

#### 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 (**9**). Later studies showed that the use of BV progeny over a series of passages in the TN-368 cell line would increase the

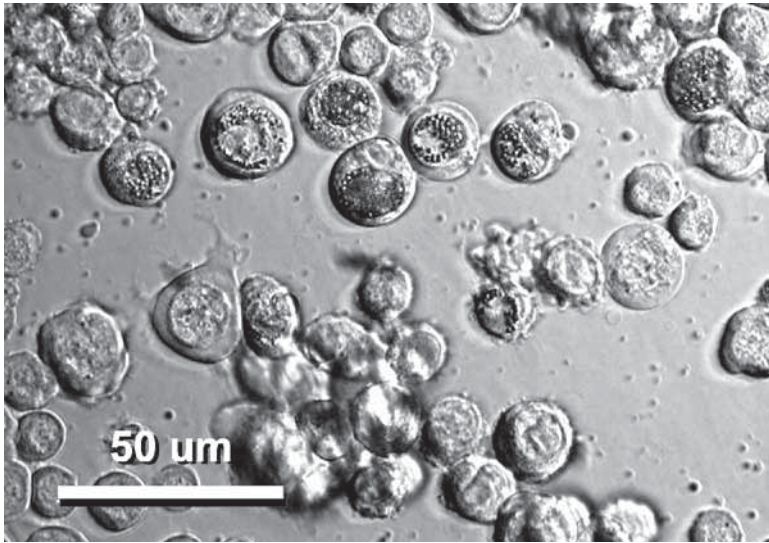


Fig. 6. IPLB-Sf21AE cell line infected with *Autographa californica* multiple nucleopolyhedrovirus 72 h postinfection. Differential interference contrast microscopy.

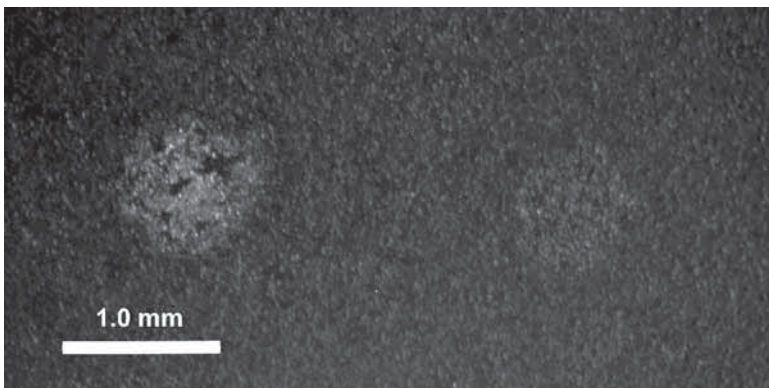


Fig. 7. MP and FP plaques of *Autographa californica* multiple nucleopolyhedrovirus in Tn-368 \*cells stained with neutral red.

proportion of FP (10). See Krell (11) for a review of this and other passage effects. The effect can be reversed by feeding OBs to a susceptible insect host (12) or using OB-derived virus (ODV) to infect cell cultures (13). 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 (14).

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 (15) could isolate viable virus from soil after more than 4 yr. Lyophilization and storage in airtight containers (even using sealed glass ampoules such as that are previously described 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 (16) simply placed cell culture supernatant containing *A. californica* multiple nucleopolyhedrovirus 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 (17) and is still available from a variety of commercial sources. Grace's medium using Hink's additions (18) are also commonly available. TC-100 (originally described in the literature as BML-TC/10) (19) is a modification of Grace's medium in which a few ingredients subsequently determined to be nonessential or detrimental have been omitted. IPL-41 is a medium developed specifically for IPLB-Sf21AE cells (20) 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). Although 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<sup>2</sup>, and so on, sizes. The author's preference is to use 25-cm<sup>2</sup> 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 and, in my experience, only personal preferences should be the deciding factor. The author has used BD Falcon®, Corning®, CoStar®, Greiner Cellstar®, and Nunclon® tissue culture labware with no discernable differences in the growth of lepidopteran cell lines. 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. Although 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 [21]), 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, 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., ME, ICD, PCI, and 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 (22) and two-dimensional gel electrophoresis of cellular proteins (23) for identifying insect cell lines. If you routinely use some of these techniques in your laboratory, 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. The author has not found this to be an issue for the cultures the authors maintains and, in fact, the author feels 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, because the cells themselves are chilled prior to subculturing, keeping the medium cold is probably advantageous.
5. The author uses a small trashcan lined with an autoclave bag 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 spectrophotometer for determining the activity in the sample. The author generally omits this step because he has learned from experience how much activity can be expected from insect cells for these enzymes. Typically, the author uses a full 1  $\mu\text{L}$  of extract per lane for ME, ICD, and PGM and 1  $\mu\text{L}$  of a 1:2 dilution (using the stabilization buffer for the dilution) 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. Some reports have indicated that cells lose their ability to replicate baculoviruses (24). This almost certainly is cell line- or virus-dependent and may vary by investigator, being dependent of how carefully the cultures are handled. In my experience, cells become quite stable in their properties by the time they have been maintained in culture for 1 yr. 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 owing 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 so the first time you thawed cells, the event would have already occurred. Unless you are establishing your own cell lines (as described in Chapter 7), the cells you obtain have likely already been through a freeze.
9. Although LN<sub>2</sub> is a useful tool for storing cell cultures, the reader should be careful in its use. At –186°C, exposure to LN<sub>2</sub> immediately freezes flesh, causing severe burns. Additionally, the Dewar should be kept in a well well-ventilated room because nitrogen is continuously being released and can replace the oxygen in an enclosed space. The author strongly encourages the reader to read the material safety data sheet on LN<sub>2</sub> for more details about this material.
10. For most cell lines the author works with, this represents between 1 and 2 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.
11. 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.

12. Researchers may be tempted to use plastic freezing vials. These can be used if the vials are only stored in the vapor phase of the LN<sub>2</sub> Dewar. This is not practical with the containers I use at my facility.
13. The glass-sealing apparatus the author uses 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 the author recommends keeping them covered as much as possible until they are sealed. As an alternative to the sealing apparatus, the author has successfully sealed ampoules by hand using a gas-air torch. For this procedure, the author temporarily attaches a glass rod to the tip of the ampoule by melting the glass on both. Then, the ampoule stem is heated about a centimeter lower until the glass melts. With a smooth motion, the rod is twisted and pulled to form the seal. This procedure can be very effective but the author recommends practicing on empty ampoules until you become proficient. *Use of eye protection is very important when working with sealed glass ampoules.* If the interior is heated after the seal is complete, the ampoule can explode. This is also a hazard when removing ampoules from LN<sub>2</sub> because 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.
14. The controlled-rate freezing apparatus lowers the temperature a 1°C per minute. As an alternative, the author has successfully placed the ampoules in a small Styrofoam box and placed it in a -80°C ultralow freezer overnight. The ampoules are then transferred to the LN<sub>2</sub> for storage.

## Acknowledgments

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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## Small-Scale Protein Production With the Baculovirus Expression Vector System

Steve Harwood

### Summary

Improved methods of baculovirus cloning and insect cell culture and their commercialization have made the use of the baculovirus expression vector system (BEVS) a routine tool for the production of preparative quantities of recombinant protein. This chapter outlines basic techniques for small-scale protein production using the BEVS, including protocols for expression from adherent and suspension insect cell cultures, titer estimation, and expression optimization.

**Key Words:** Baculovirus expression vector system; BEVS; protein expression; titer estimation.

### 1. Introduction

Recombinant protein expression in insect cells is often an excellent compromise between bacterial expression systems and protein expression in mammalian cells. Although bacterial expression systems have lower culture costs and often result in high yields, many eukaryotic proteins are not correctly folded or posttranslationally modified in *Escherichia coli*. In mammalian cells on the other hand, recombinant proteins are generally expressed at lower levels, although the proteins produced tends to be correctly folded, posttranslationally modified, and biologically active. Baculovirus-infected insect cells, which are collectively referred to as the baculovirus expression vector system (BEVS), perform most posttranslational modifications performed by mammalian cells and are capable of expressing recombinant proteins with high yields. Although the posttranslational modifications present on proteins produced in insect cells may not be identical to those produced in mammalian cells, biological activity is

often not compromised (**1**) (*see* Chapter 17). With the development of convenient vector systems over the last 15 yr, the BEVS has become a frequently used tool for recombinant protein expression.

For the purposes of this chapter, small-scale expression will be defined as any culture volume, from adherent culture in wells of a 96-well plate to shake or spinner cultures of up to 250 mL. Protein production protocols are readily scaleable, but in suspension cultures greater than 250 mL, parameters important for large-scale cultures such as oxygen levels, nutrient depletion, and accumulation of waste products become increasingly important (*see* Chapter 11). Specialized equipment needs for small-scale protein expression experiments are minimal, requiring only an incubator, shaker, inverted microscope, and a sterile working area. Depending on the aims and scope of an experiment, small-scale expression as defined here, may be all that is required. Although yields vary widely, 0.5–5 mg yields can commonly be obtained from a single 50-mL shake flask. Multiple flasks are easily accommodated; thus, small-scale insect cell cultures can provide sufficient protein for crystallization, activity assays, antigen production, etc.

There are a number of parameters that should be considered for successful BEVS protein expression. Before creating a recombinant baculovirus, define how the virus will be used, what protein will be expressed, and how the protein will be used/purified. Understanding these issues will help select the expression vector best suited for a particular purpose. There are a number of vector systems available (*see* Chapter 3). For small-scale protein production, expression systems that do not require plaque purification are desirable (*see* Chapter 4); however, irrespective of the vector system chosen, isolation of an isogenic virus by plaque purification is recommended for large-scale cultures. The choice of whether a protein should be expressed intracellularly or secreted into the media should be based on the protein being expressed, the manner in which it will be used, and the method of purification to be employed. Genes may be cloned into a vector that provides a secretion signal or cloned such that their native secretion signal is used. Insect cells recognize and cleave most eukaryotic secretion sequences efficiently, although not all proteins with a secretion signal will be correctly processed (**1,2**). Another important consideration is whether or not a fusion tag is desirable, and if so, which tags to use. Epitope tags (e.g., HA or V5) simplify monitoring of expression by Western blotting, although purification tags simplify downstream purification. Protein expression or biological activity may be affected by the presence of extraneous sequence or whether the additional sequence is placed on the N- or C-terminus of the protein (**3**). Use of a C-terminal tag is convenient because the native version of the protein can easily be cloned and expressed concurrently with the tagged version, simply by inclusion of a stop codon. If possible, nonnative

sequences should be located away from domains known to be critical for protein function. Every protein is unique; there are no generally applicable rules that govern the best tag or tag placement. If possible, multiple approaches should be tried simultaneously.

Although the polyhedrin promoter is most commonly used for recombinant protein expression with the BEVS (and should be tried first for most applications), promoters that result in earlier expression or lower expression levels can sometimes be useful (*see* Chapter 3). Finally, stable transfection methods ([4]; *see also* Chapter 15) can sometimes be superior to baculovirus infection, particularly for secreted proteins or proteins for which solubility is problem.

This chapter outlines general procedures for the small-scale culture, infection, titring, expression optimization, and harvest of insect cells for protein production.

## 2. Materials

### 2.1. Cell Culture and Baculovirus

#### 2.1.1. Cells and Media

The most common cell lines used for small-scale protein expression using baculovirus are *Spodoptera frugiperda* Sf-9 and Sf-21, and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5, sold as HighFive™ by Invitrogen). The Sf-9 and Sf-21 cell lines are most useful for transfection, virus amplification, and protein expression, whereas Tn-5 cells are primarily used for protein expression. For expression of complex glycoproteins, the SfSWT-1 cell line (Mimic™, Invitrogen) can be used to produce sialylated glycoproteins (*see* Chapter 17). Ultimately, the optimal cell line for a given protein should be established empirically. Cell lines and culture methods are discussed in Chapters 6, 7, and 9. Cells grown in serum containing or serum-free media (SFM) are suitable for small-scale protein expression experiments, although use of SFM simplifies purification of secreted proteins. For experiments, cells may be infected as adherent cultures or in suspension. Adherent cultures are useful for preliminary experiments such as initial checking for recombinant protein expression or multiplicity of infection (MOI) optimization, whereas suspension cultures are more scalable and are better choice to obtain sufficient protein for purification. Suspension cultures are also better suited for developing conditions for larger-scale expression studies.

#### 2.1.2. Baculovirus

General considerations for choosing and constructing baculovirus vectors are discussed in Chapters 3 and 4. Ideally, virus stocks should be recently titered and of low passage. Serial passage of viral stocks results in the accumu-

lation of defective interfering particles at the expense of functional virus. Maintain seed and master stocks (Chapter 9) to avoid using baculovirus stocks that have been passaged indefinitely.

## **2.2. Generic Six-Well Plate Experiment**

1. Six-well plates.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium (*see* **Notes 1** and **2**).
3. Virus stock to be tested.
4. Positive control virus (preferably one that expresses a protein recognized by the same antibody used to detect the protein to be studied).
5. Antisera specific for protein being expressed (*see* **Note 3**).
6. 4X Sodium dodecyl sulfate (SDS) sample buffer: 0.25 M Tris-HCl (pH 6.8), 10% glycerol, 0.04% (w/v) bromophenol blue, 8% SDS, add water to 9.6 mL final volume. Combine in a screw cap conical tube and store at 4°C. Prior to use, add 400  $\mu$ L (or 4% v/v)  $\beta$ -mercaptoethanol to sample buffer before use.
7. Materials for SDS-polyacrylamide gels (PAGE) and Western blot.

## **2.3. Optimization of Infection Parameters for Small-Scale Expression**

1. 24-Well plates.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Virus stock to be tested.
4. Antisera specific for protein being expressed.
5. 4X SDS sample buffer.
6. Materials for SDS-PAGE and Western blot.

## **2.4. 50-mL Culture for Protein Production**

1. 250-mL Erlenmeyer tissue culture flasks (*see* **Note 4**).
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Virus stock to be tested.
4. Positive control virus.
5. Antisera specific for protein being expressed.
6. PBS: 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 10.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 140 mM NaCl, 40 mM KCl, adjusted to pH 6.2 with 1 N NaOH.
7. 4X SDS sample buffer.
8. Native lysis buffer: 25 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1% NP40 (*see* **Note 5**).

## **2.5. Titering Virus by Limiting Dilution**

1. 96-Well plates.
2. Exponential phase cells (*see* Chapter 1) grown and adapted to appropriate medium.
3. Optional but recommended: multichannel pipettors (2–20 and 20–200  $\mu$ L) and repeating pipettor (capable of accurately dispensing 10  $\mu$ L).

4. Virus stock to be tested.
5. Inverted microscope with  $\times 4$  and 10 objectives.

## 2.6. Cell Lysis

1. Native lysis buffer: 25 mM sodium phosphate, 150 mM NaCl, 1% NP40, pH 7.0. Protease inhibitors (phenylmethylsulfonyl fluoride [1 mM], leupeptin [5  $\mu\text{g}/\text{mL}$ ], pepstatin [1  $\mu\text{M}$ ], E64 [2  $\mu\text{g}/\text{mL}$ ]) can be added alone or in combination if required (5–7) (see Note 5).

## 3. Methods

### 3.1. Generic Six-Well Plate Experiment

This basic experimental outline is useful for checking protein expression from new virus constructs.

1. Plate healthy exponential growth phase cells (see Chapter 1) from liquid culture (see Note 2). Count and plate  $0.75\text{--}1.0 \times 10^6$  cells in a volume of 2 mL per well in a six-well plate. Evenly distribute the cells by gently rocking the plate back and forth 5–10 times, or place on a rocking platform for 2 min. Allow the cells to attach at least 1 h.
2. Add a volume of recombinant virus stock to provide a MOI of 10 to one well (see Note 6). In a second well, infect with a positive control virus at the same MOI. Leave cells in one well uninfected. The volume of virus stock to add to obtain an MOI of 10 is calculated as follows (assuming  $10^6$  cells per well and a virus stock with a titer of  $5 \times 10^8$  plaque forming units [pfu]/mL):  $(10^6 \text{ cells} \times 10 \text{ pfu/cell}) / 5 \times 10^8 \text{ pfu/mL} = 0.02 \text{ mL}$ , or 20  $\mu\text{L}$  virus stock.
3. Gently mix the solution by hand or place on rocking platform for 2 min.
4. Place the plate in a humidified incubator set to 27°C (or if using an incubator without humidity control, the plate may be sealed in a plastic bag with a moistened paper towel).
5. At 72 h postinfection (pi), harvest cells and media. Analyze by SDS-PAGE (Subheading 3.5.) (see Note 7).

### 3.2. Optimization of Infection Parameters for Small-Scale Expression

The MOI and time of harvest are important for effective protein expression and are best established empirically. MOI and time of harvest are interdependent and are also affected by cell type, culture conditions, and the protein being expressed. MOIs of 2–10 are generally best for a synchronous infection (see Note 6). Higher MOIs can be often detrimental to protein expression. A simple factorial experiment can be used to determine the optimal MOI and time of harvest. Varying amounts of virus are added to cells in rows of a 24-well plate. The cells in each row are then harvested at various times pi. The harvested cells/media are then analyzed for protein expression by Western blot. The results

obtained can then be used as an optimized starting point for larger scale expression experiments.

1. Plate desired cell line in a 24-well plate at  $2.0 \times 10^5$  cells/well by diluting healthy exponential phase cells to  $10^6$  cells/mL in the appropriate medium and then adding 200  $\mu$ L of these diluted cells in each well. Allow cells to attach at least 1 h.
2. During the attachment step, plan a dilution series for the virus stock to be optimized. Add virus stock equivalent to 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 pfu/cell (see **Note 8**). Array five 1.5-mL tubes in an appropriate rack. Dilute the virus stock to  $8 \times 10^7$  pfu/mL in 500  $\mu$ L, place in the first tube. Pipet 200  $\mu$ L medium into each of the other four tubes. Transfer 200  $\mu$ L diluted virus stock from the first tube to the next tube and mix well. Transfer 200  $\mu$ L from tube 2 to tube 3 and mix well. Repeat for tubes 4 and 5.
3. After the cells have attached, add the virus dilutions as follows: viewing the 24-well plate as a grid of four rows (horizontal dimension) and six columns (vertical dimension), transfer 50  $\mu$ L from the first tube to the cells in each well of column 1. Transfer 50  $\mu$ L from tube 2 to each well in column 2. Repeat for tubes 3–5. Leave the cells in the wells of column 6 noninfected.
4. After adding the virus stocks to the plate, gently mix, and place in the incubator.
5. Harvest the cells in row one 48 h pi. Harvest row 2 at 72 h pi. Harvest row 3 at 96 h pi, and row 4 at 120 h pi. At each time point, harvest the cells and medium using a stream of medium (see **Notes 9 and 10**). Remove cells to 1.5-mL tubes. Keeping the cells in suspension, remove 45  $\mu$ L of suspension to fresh tubes. Add 15  $\mu$ L of 4X SDS sample buffer. Heat to 70°C for 5 min. Store samples at –80°C for later analysis.
6. After all time points have been collected, analyze by SDS-PAGE and Western blotting (**Subheading 3.5**). Load 10  $\mu$ L of lysed cell suspension per lane, transfer to PVDF membranes, and perform Western blot with an appropriate antibody.

### 3.3. 50-mL Culture for Protein Production

This protocol is a basic scaleable method for suspension cell production of recombinant protein. It is generally scaleable to 1 L size, and can be adapted to spinner cultures (see **Note 4**).

1. Dilute exponential phase Sf-21 cells to  $2.0 \times 10^6$  cells/mL.
2. Add a volume of virus stock that results in an MOI of 5.0 (or an optimal MOI previously determined). Example: 50-mL culture  $\times$  ( $2 \times 10^6$  cells/mL)  $\times$  5 pfu/cell /  $5 \times 10^8$  pfu/mL = 1 mL virus stock.
3. At 72 h pi (or optimized harvest time) collect cells to 50-mL conical centrifuge tube.
4. Centrifuge cells at 3000g for 10 min. Remove media and transfer to another tube.
5. If the protein is intracellular, then discard medium and wash the cell pellet twice with PBS. Flash freeze and store at –80°C.

6. If the protein is known to be secreted, then collect the supernatant and transfer to another tube. Centrifuge medium at 30,000g for 1 h to remove virus particles and cellular debris. Collect supernatants, flash freeze, and store at  $-80^{\circ}\text{C}$ .

### 3.4. Titering Virus by Limiting Dilution

The two most common methods for titering baculovirus are the plaque assay and limiting dilution. The plaque assay is discussed in Chapter 4; also note that alternative methods of virus titering are discussed in Chapters 5, 11, and 21. A protocol for limiting dilution is presented here. Titering virus by limiting dilution requires approx 7–10 d. Each virus stock to be titered is serially diluted in 10-fold series and added to cells in wells of a 96-well plate.

1. Dilute exponential growth phase Sf-21 cells to  $2 \times 10^5$  cells/mL in medium. Dispense 100  $\mu\text{L}$  cell suspension to each well of a 96-well plate. Allow cells to attach 1 h. After attachment, cells should appear 25–50% confluent (*see Note 11*).
2. While cells are attaching, serially dilute each virus stock to be tested (*see Note 12*). Array nine 1.5-mL centrifuge tubes in a rack. Add 990  $\mu\text{L}$  medium to tubes 1 and 2, then 900  $\mu\text{L}$  medium to tubes 3–9.
3. Add 10  $\mu\text{L}$  of the virus stock to be titered to tube 1, vortex (dilution =  $10^{-2}$ ).
4. Transfer 10  $\mu\text{L}$  from tube 1 to tube 2 and vortex (dilution =  $10^{-4}$ ).
5. Transfer 100  $\mu\text{L}$  from tube 2 to tube 3, vortex (dilution =  $10^{-5}$ ).
6. Transfer 100  $\mu\text{L}$  from tube 3 to tube 4, vortex (dilution =  $10^{-6}$ ).
7. Repeat **step 6** for tubes 4–8 (dilutions of  $10^{-7}$  through  $10^{-10}$ ). Leave medium only in tube 9.
8. Using a repeating pipettor (*see Note 12*), withdraw 120  $\mu\text{L}$  of diluted virus stock from tube 2, and dispense 10  $\mu\text{L}$  to each well in row 1 of the plate. Gently agitate the plate to mix.
9. Withdraw 120  $\mu\text{L}$  from tube 3 and dispense 10  $\mu\text{L}$  to each well in row 2. Similarly, dispense 10  $\mu\text{L}$  to each well of row three from tube 4, 10  $\mu\text{L}$  to each well of row 4 from tube 5, and so on. Repeat for the remaining tubes. Tube 9 should have media only, leaving the cells in row 8 noninfected.
10. Gently mix the virus dilutions in the plate with a circular motion, and then put the plate(s) in a sealed plastic bag containing a wet paper towel (to keep the plate from drying out) and place in the incubator. Allow cells to become completely infected (5–7 d).
11. After 5–7 d, analyze the plate and score each well for signs of infection. Wells receiving at least one productive virus particle become infected and are scored positive, whereas wells receiving no productive virus particles remain uninfected and are scored negative (*see Note 13*). Results are tabulated as numbers of infected wells at each dilution. *See Table 1* for a sample data set. A spreadsheet for performing this calculation is shown in **Fig. 1**. Both are taken from O'Reilly et al. (6). The tissue culture infective dose ( $\text{TCID}_{50}$ ) is the virus dose that causes 50%

**Table 1**  
**Sample Data Set for Calculating Virus Titer by Limiting Dilution**

Dilution	Infected	Uninfected	Cumulative		Percent infected
			Infected	Uninfected	
10 <sup>-4</sup>	12	0			
10 <sup>-5</sup>	12	0			
10 <sup>-6</sup>	12	0	22	0	100
10 <sup>-7</sup>	9	3	10	3	77
10 <sup>-8</sup>	1	11	1	14	6.7
10 <sup>-9</sup>	0	12	0	26	0
10 <sup>-10</sup>	0	12			

of the cultures to become infected. The number of wells infected or uninfected is summed cumulatively and thus, from **Table 1**, the TCID<sub>50</sub> corresponds to a dilution that must be between 10<sup>-7</sup> and 10<sup>-8</sup>. The TCID<sub>50</sub> is estimated by interpolation as the proportional distance (PD) between the upper response and 50%:

PD = (A–50)/(A–B), where PD is the proportional response, A is the percent response greater than 50% and B is the percent response less than 50%. As per this example,

$$PD = (77-50)/(77-6.7)$$

$$PD = 27/70.3 = 0.38$$

The dose that would give a 50% response is then calculated as:

Log<sub>10</sub>(TCID<sub>50</sub>) = log of the dilution giving a response greater than 50%—the PD of that response, or Log<sub>10</sub>(TCID<sub>50</sub>) = -7.0 - 0.38 = -7.38.

Therefore, TCID<sub>50</sub> = 10<sup>-7.38</sup>. The virus titer is the reciprocal of the TCID<sub>50</sub>, expressed in mL of virus dilution added, i.e., (1/[10<sup>-7.38</sup>])/0.01 mL or 2.4 × 10<sup>9</sup> TCID<sub>50</sub>/mL. Based on the Poisson distribution (*see* Chapter 1), the TCID<sub>50</sub> units can be converted to pfu by multiplying the TCID<sub>50</sub> by 0.69, so the titer of this virus in pfu is 0.69 × (2.4 × 10<sup>9</sup>) or 1.7 × 10<sup>9</sup> pfu/mL (*see* **Note 14**). *See* **Fig. 1** for a spreadsheet that performs this calculation.

12. Optional (*see* **Note 15**): after 4 d, plate a fresh 96-well plate of Sf-21 cells as in **step 1**. Allow cells to attach 1 h.
13. Using a multiwell pipettor, transfer 10 μL of supernatant from each well of the first plate to the wells of the new plate, changing tips for each row.
14. After 5 d, examine the cells under the microscope. Score wells showing any signs of infection as positive and analyze the data as previously described.

### 3.5. Analysis of Protein Production: General Considerations

Without an activity assay, protein expression can be monitored by Western blotting. SDS-PAGE of a cell extract equivalent to 5000 cells (approx 10 μg

	A	B	C	D	E	F	G	H	I	J	I	
1	Name:											
2	Date:											
3	Virus stock (Passage):											
4	Cell line:											
5												
6		<b>Number of Infected Wells</b>	<b>Number of Uninfected Wells</b>	<b>Total Number Infected</b>	<b>Total Number Uninfected</b>	<b>% Total Infected</b>	<b>Above 0.50</b>	<b>% Above 0.5</b>	<b>% Below 0.5</b>	<b>Log Dilution Above 50%</b>	<b>% Below 0.5</b>	
7	<b>Dilutions</b>											
8												
9	1.00E-04	12	0	46	0	100.00%	TRUE	0.00%	0.00%	0.00	0.00%	
10	1.00E-05	12	0	34	0	100.00%	TRUE	0.00%	0.00%	0.00	0.00%	
11	1.00E-06	12	0	22	0	100.00%	TRUE	0.00%	0.00%	0.00	0.00%	
12	1.00E-07	9	3	10	3	76.92%	TRUE	76.92%	0.00%	-7.00	0.00%	
13	1.00E-08	1	11	1	14	6.67%	FALSE	0.00%	6.67%	0.00	33.33%	
14	1.00E-09	0	12	0	26	0.00%	FALSE	0.00%	0.00%	0.00	0.00%	
15	1.00E-10	0	12	0	38	0.00%	FALSE	0.00%	0.00%	0.00	0.00%	
16	Num. Wells	12						76.92%	6.67%	-7.00	33.33%	
17	mL/well	0.01										
18												
19	Prop. Dist.	0.383										
20	Log TCID	-7.383										
21	TCID50	4.14E-08										
22	1/TCID50	2.42E+07										
23	<b>TCID50/ml</b>	2.42E+09										
24												
25	pfu/ml	1.67E+09										
26	<b>I.U./ml</b>	1.67E+09										

Fig. 1. Excel spreadsheet for calculating titers by limiting dilution. Reprinted from ref. 9 with permission from Oxford University Press.

protein) is usually sufficient to detect most proteins by Western blot. The details of SDS-PAGE and Western blotting will not be discussed. Commercial precast SDS PAGE gels are widely available, are cost-effective, and are recommended for all but the most budget-constrained laboratories. To simply check for expression, cells and medium can be lysed together directly into SDS-PAGE sample buffer for analysis. Alternatively, cells and medium can be separated by centrifugation (3000g, 10 min) and denatured separately in SDS sample buffer. For most purposes, it is desirable to lyse cells in a manner that preserves structure and biological activity. There is no universal buffer applicable for all proteins. Buffers should be chosen that are compatible with the purification method or activity assay that will be employed. Two basic lysis methods are presented, but ultimately the lysis method employed should be guided by how the protein will be used/purified.

### 3.5.1. Lysis in SDS-PAGE Sample Buffer

1. Add one-third volume of 4X-sample buffer to the sample.
2. Place in boiling water bath for 5 min.
3. Centrifuge at 14,000g for 5 min.
4. Load 10  $\mu$ L sample supernatant per lane.

### 3.5.2. Native Lysis

1. Consider how protein will be purified or used. Adjust the sample buffer based on how the protein will be used (*see Note 5*).
2. For cellular samples, add volume of native lysis buffer equal to 1/10 volume of the original culture volume from which the sample was obtained. Rotate cells on a rotating platform at 4°C for 15 min. Remove coarse debris by centrifuging at 3000g for 10 min. Transfer the supernatant to high speed centrifuge tubes and spin at 30,000g for 30 min. To ensure that the protein of interest is not lost, save aliquots of pellet and supernatant from each spin to check with SDS-PAGE. Flash freeze the remaining supernatant for storage at -80°C, or go to purification or assay.
3. For secreted protein samples, spin out cell debris at 3000g for 10 min. Remove supernatant to a high speed centrifuge tube, clarify supernatant by centrifugation at 30,000g for 30 min. Save aliquots to separate tubes for SDS-PAGE analysis. Flash freeze the remainder.

## 4. Notes

1. General considerations: any protocol for protein expression should be considered a starting point for further optimization. There are tremendous differences between cell lines and viruses, as well as between proteins. Whenever possible, vary conditions (medium, cell type, fusion tag placement, MOI, time of harvest, purification strategy, and so on) and pursue multiple strategies as time

and resources allow. Positive and negative controls save time by allowing interpretation of experiments that gave unexpected or negative results.

2. Healthy, actively growing cells are critical for efficient virus infection and protein production. Cells are more easily counted and dispensed from liquid cultures than from attached ones. Cell viabilities are generally higher and more consistent. Maintenance of 50-mL liquid cultures is usually adequate for most small experiments and can be scaled easily should more cells be needed. After cells have attached to the plate, they should appear 60–80% confluent.
3. A key advantage of using an epitope tag is the availability of specific antisera to any protein expressed in the vector.
4. As the culture volume is increased between 50 mL and 1 L, it becomes more likely that oxygen levels, nutrients, or waste products may limit productivity. In general, tissue culture flasks or spinners should be filled to no more than 20–25% of the volume of the flask. For larger volumes, consult Chapter 11.
5. The basic native lysis buffer can be supplemented in a variety of ways to suit the purification method or application such as addition of glycerol,  $\beta$ -mercaptoethanol (or dithiothreitol), more or less salt, different detergents, different buffers, protease inhibitors, and so on. Consult the literature available for each protein to be expressed and choose a buffer consistent with how the protein will be used.
6. MOIs of 2–10 are common for synchronous infections. If the virus has not been titered, use the other wells to infect with different volumes of virus stock (i.e., 1, 10, and 50  $\mu$ L). These are only guidelines (*see Subheading 3.2.* to optimize infection parameters). An adsorption step during which cells are exposed to virus in a reduced volume for 1–2 h may improve infection kinetics although is not usually necessary for simple expression experiments (8). However, the inocula should be replaced after a 1- to 2-h attachment period if the volume of virus stock (spent media) added represents more than 20% of the total volume of the well. Many researchers routinely resuspend the cells in fresh medium approx 1–4 h following baculovirus infection in order to prevent nutrient depletion and toxic byproduct accumulation.
7. If the protein of interest was not detected by Western blot but the control protein was detected, the most likely explanations are: (1) the virus is too low in titer (examine cells for signs of infection) or (2) gene cloned improperly (check reading frame between insert and fusion partners).
8. If the titer of the virus stock is not known, then infection parameters can be optimized based on the volume added. That is, add varying amounts of virus stock to the wells, e.g., 1, 3, 10, 30, and 100  $\mu$ L to the wells in each column.
9. The times suggested are a starting point. After the broad timing of expression is set, it is possible to optimize time of harvest more precisely by collecting samples at 8–12 h intervals.
10. The optimal time of harvest may be different for a secreted protein than the same protein expressed intracellularly. If the protein is secreted, then separate the medium from the cells (centrifuge at 3000g) and assay separately.

11. The ideal cell density should allow completion of multiple rounds of virus replication before the cells reach confluency, while still allowing the cells to grow in exponential growth phase in the absence of virus. This simplifies scoring of infected wells.
12. Accurate pipetting is critical for accurate results. Change tips between dilutions. Take care to dispense all of the solution from each tip by just touching the tip to the meniscus of the fluid in the next tube. Use of a repeating pipettor is strongly recommended for dispensing dilutions onto the plate.
13. A well is counted as positive if there are any infected cells detected. If no wells of the plate show evidence of infection even at the highest MOI, then the virus titer is too low to be useful. On the other hand, if all of the wells appear to have infected cells, then there may be carry-over from lower to higher dilutions.
14. Reasonable titers are in the range of  $10^7$ – $10^9$ , with titers in the higher end of this range being best. Values over  $10^{10}$  are likely erroneous.
15. The optional passage of virus is useful to confirm infection in wells that may have only a low level of infection.

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

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### Summary

The production of recombinant proteins using the baculovirus expression vector system 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 online monitoring and bioreactor control, which have a significant impact on the overall success of the process, are provided, including advanced online monitoring of physiological parameters such as biovolume and respiration activity for batch and fed-batch insect cell cultures along with their role in operating high cell density cultures.

**Key Words:** Bioreactor; large-scale; recombinant protein expression; fed-batch; high cell density; insect cell; lytic system; baculovirus; on-line monitoring; serum free.

### 1. Introduction

The baculovirus expression vector system (BEVS) is one of the most commonly used recombinant gene expression systems (1,2). The BEVS utilizes a recombinant baculovirus in which a foreign gene has been inserted into a non-essential region of its genome used in conjunction with an insect cell host to produce a recombinant protein. It is a versatile system and genes from different sources, ranging from bacterial to human proteins, can be expressed using this system. The use of this protein expression system only involves engineering the baculovirus and not an entire cell line. This significantly reduces the time required to complete the process from cloned gene to protein from months to a

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

few weeks. The expression levels are generally high and the protein is produced with most posttranslational modifications and is usually biologically active.

Because the BEVS is a lytic system, it can be used to express proteins even when their functions and characteristics are not fully known, thereby making it invaluable in a wide variety of applications spanning the life sciences, including studies on gene regulation, protein expression, and drug discovery research, and the commercial production of virus-like particles, vaccines, and therapeutics. Thus, this expression system has the potential to be the enabling technology for the proteomics era by satisfying the demand of the genomics industry for rapid protein production.

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 cultures. The use of media additives, e.g., Pluronic® F-68, a polyol detergent that protects cells against hydrodynamic shear, and supplements, e.g., yeastolate, that can be used in insect cell media to replace serum, have facilitated the scale up of the BEVS in agitated bioreactors. Insect cells have been grown in different bioreactor types operated either in batch, fed-batch, or perfusion modes (3–7).

In this chapter, the different elements comprising the insect cell-BEVS process will be considered as they apply to large-scale protein production. To successfully produce protein using the insect cell-BEVS in a bioreactor, it is important to consider the key components of the system and their interdependence. These include the insect cell host, the medium, the recombinant baculovirus carrying the gene of interest, and the bioreactor operating conditions. It is important to keep in mind that each of these elements can influence the final product yield and bioactivity. Some of the specific items to consider include:

1. The physiological status of the cells and the growth phase at infection can affect the infection process.
2. The nutrient and byproduct concentrations in the medium and the bioreactor conditions can have a profound effect on cell physiology and metabolism.
3. The virus itself is another critical factor. The method of generating the recombinant virus and the promoters used are important aspects to be considered.

## 2. Materials

### 2.1. Cell Culture

1. A list of potential host insect cells is given in Chapter 6. The most commonly used insect cells for recombinant protein production are the continuous cell lines derived from *Spodoptera frugiperda* (i.e., Sf-9 and Sf-21) and *Trichoplusia ni*

(Tn-368 and BTI-Tn-5B1-4 [Tn-5] that is commercially available as High Five™ from Invitrogen, Carlsbad, CA). This chapter focuses on the use of Sf-9 cells in suspension culture using serum-free medium for the large-scale production of proteins in bioreactors.

2. Serum-free medium. Several commercial preparations of serum-free media are available, many of which are discussed in Chapter 8 (*see also Note 1 and ref. 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.
3. Shake flasks (Erlenmeyer flasks, Corning, 125, 250 mL, and so on) are used for Sf-9 cell growth.
4. Inverted light microscope.
5. Hemacytometer and cover slips (Hauser Scientific, Horshaw, PA).
6. 0.4% Trypan blue solution (e.g., Sigma-Aldrich, St. Louis, MO).

## 2.2. Baculovirus Amplification and Quantification

1. Baculovirus stock (*see Note 2*).
2. Beckman, L-8 Ultracentrifuge (Beckman, Inc., Palo Alto, CA).
3. Ultrafiltration membrane 50-kDa low protein-binding and ultrafiltration apparatus (Pall Filtron Corporation, Canada).
4. Diafiltration apparatus.
5. Low protein-binding 0.22- $\mu$ m membrane (Corning, Inc., Corning, NY).
6. Flow cytometer EPICS XL-MCL (Beckman Coulter, Inc., Miami, FL).
7. Sterile phosphate-buffered saline (PBS) without calcium and magnesium.
8. Nonsterile screw-caped 1.5-mL tubes such as Sarstedt microtubes (Starstedt, Nümbrecht, Germany) (*see Note 3*).
9. Paraformaldehyde (ICN Biochemicals, Inc., Aurora, OH) 5% solution prepared in PBS.
10. Dry ice/ethanol or liquid nitrogen for quick sample freezing.
11. Triton X-100: 10% solution in PBS.
12. SYBR Green I (S-7585) (Molecular Probes, Inc., Eugene, OR).
13. Water bath at 80°C.
14. Polystyrene tubes for fluorescence-activated cell sorting (FACS) analysis.
15. Flow-set Beckman Coulter (cat. no. PN 6607007).

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

1. Agitated bioreactor vessel with height to diameter ratio close to one.
2. Marine, scoping, or pitched blade impellers.
3. Agitation speed controller able to control over a wide range of agitation speeds (e.g., 50–300 rpm).
4. Mass flow controllers used to regulate air flow into the bioreactor (*see Note 4*).
5. Temperature probe and controller (capable of controlling the temperature at 27°C).
6. Dissolved oxygen (DO) polarographic electrode (Ingold, Andover, MA).

7. pH electrode (Ingold).
8. Assorted accessories (cell sampler, sparger, and so on).
9. (Optional) Infrared analyzer (Servomex 1400, Norwood, MA) for measuring the CO<sub>2</sub> evolution rate.
10. (Optional) Model 214M (Aber Instruments, Ltd., Aberystwyth, UK) for biomass monitoring.

A schematic of the bioreactor setup and the associated control system is shown in **Fig. 1**. 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.

The physical features and setup of bioreactors is the same irrespective of the cell type (i.e., microbial, mammalian or insect) that is to be grown. 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.

### 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 prewarmed to 27°C) to obtain a cell density of approx  $0.5 \times 10^6$  cells/mL. Determine the cell density of the culture by performing cell counts using a hemacytometer and the Trypan blue staining method as described in **Subheading 3.1.2**. A Coulter counter may be used to determine the total cell number and the average cell diameter (*see* **Note 5**).
2. Seed Erlenmeyer flasks (*see* **Note 6**) 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. Lower volumes may result in lower viabilities owing to greater shearing of cells during shaking, whereas higher volumes may lead to oxygen limitations because of inadequate gas/liquid surface area for oxygen transfer.
4. Incubate cultures in an incubator shaker at 27°C at 110–120 rpm.
5. Continue expanding the cells to additional Erlenmeyer flasks to provide a sufficient number of cells to seed the bioreactor at approx  $0.5 \times 10^6$  cells/mL. It is critical that the cells are maintained in exponential growth phase, i.e., do not

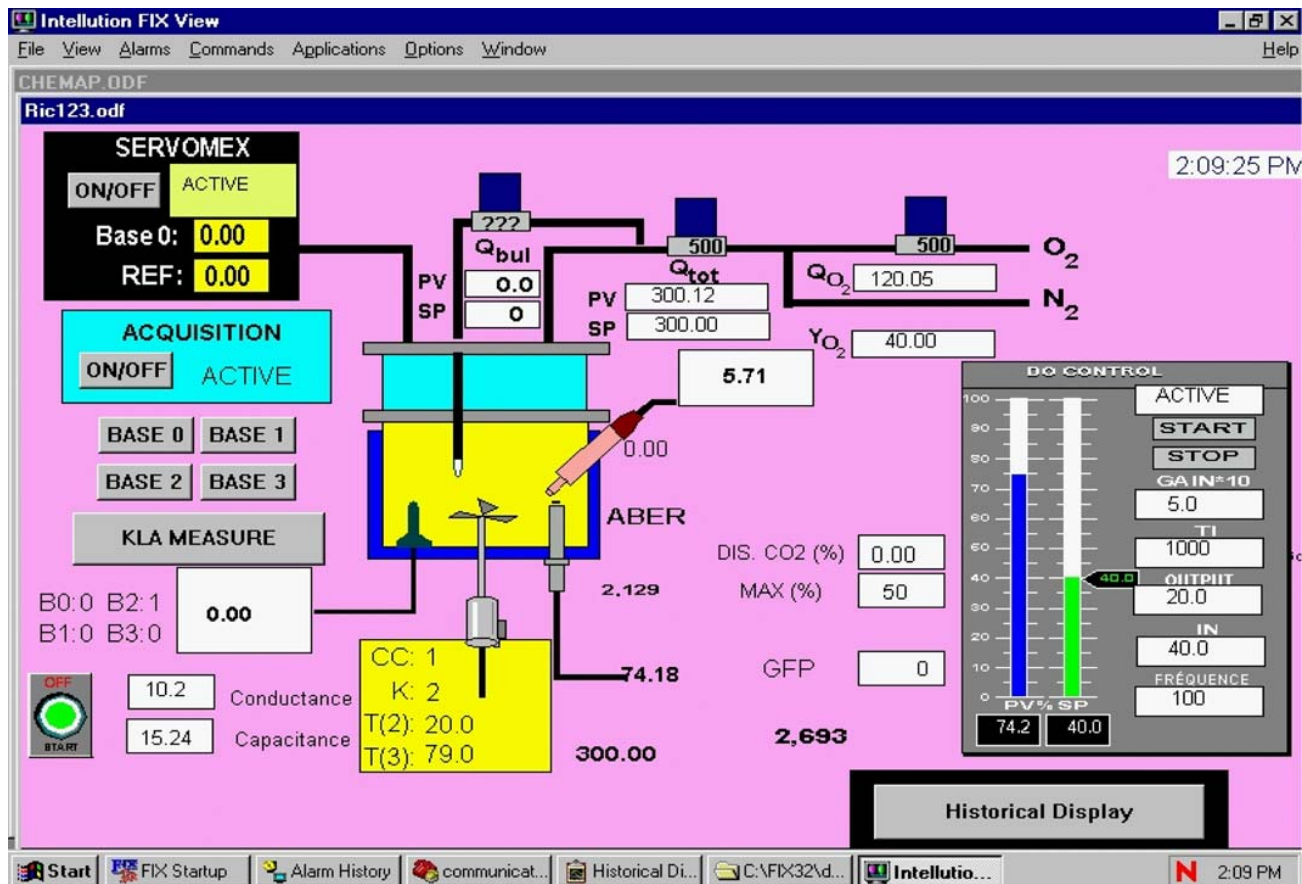


Fig. 1. Schematic representation of the bioreactor setup and associated instrumentation.

allow the cells to grow into the stationary growth phase (*see* Chapter 1). Furthermore, the cell viability should be at least 90–95%.

### 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. Although the Trypan blue method is not a direct measure of viability (it is considered to be a measure of membrane integrity), this method is the most commonly used technique to approximate the cell viability; especially in the case where infected cells are being studied. The application of this protocol is based on the assumption that viability precedes the loss of membrane integrity that is characteristic of nonviable cells. Alternatively, other counting devices, e.g., a Coulter Particle Counter, can be used to perform the cell counts (*see* **Note 7**).

The Trypan blue method is briefly described next:

1. Withdraw 0.9 mL of sample from each shake flask using sterile pipet under aseptic conditions and place in test tube.
2. Immediately return the shake flasks to the incubator.
3. Dilute the sample to have between 30 and 100 cells per 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 Pasteur pipet. 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 pipet, gently place the tip of the pipet on the slot of a clean hemacytometer and allow the suspension to fill the chamber between the slide and the cover slip.
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. Note that each of these squares has a volume of  $0.1 \text{ mm}^3$  ( $1 \times 1 \times 0.1 \text{ mm}$  [depth]).
7. The total and viable cell density can be calculated using **Eqs. 1** and **2**, respectively:

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

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

where where  $N$  = total number of stained (i.e., dead) cells counted,  $N'$  = total number of unstained (i.e., viable) cells counted,  $n$  = number of squares in which cells were counted (usually 4), and  $D$  = dilution factor.

8. The percent viability can be calculated using **Eq. 3**:

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

## 3.2. Baculovirus Amplification and Quantification

### 3.2.1. Virus Stock Amplification

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

1. Start Sf-9 cultures in shaker flasks (200–250 mL working volume in a 1-L flask) at about  $0.5 \times 10^6$  cells/mL by diluting an exponential growth phase culture (see Chapter 1). Grow the cells in a shaker incubator at 27°C at 110 rpm.
2. Determine the cell density and viability daily.
3. Add sufficient baculovirus stock to infect the cells at a multiplicity of infection (MOI) between 0.01 and 0.1 (see **Note 8**).
4. Incubate further and continue to monitor the cell density and viability infected culture by counting the viable cells. It is also useful to monitor the average cell diameter (cell size increases significantly as a result of the baculovirus infection), which is greatly simplified if a Coulter counter is used (see **Note 5**). There is a decline in the viability starting at 48–80 h postinfection (pi) depending on the MOI used (see **Note 9**).
5. Harvest the cells, when the viability of the culture is less than 90%. This typically occurs between 72 and 120 h pi.
6. Centrifuge the cell suspension at 1000g to remove cells.
7. Collect the supernatant, filter immediately through a 0.22- $\mu$ m filter. Store the viral stock at 4°C until further use for up to 6 mo (see **Note 10**).

### 3.2.2. Baculovirus Stock Concentration

The titers of viral stocks produced using routine methods are usually in the range of  $10^8$  to  $10^9$  plaque forming units (pfu)/mL. It may be desirable to have higher virus titers when using high MOIs in high cell density cultures (e.g., an MOI of 10 is commonly used to achieve a synchronous infection). This is done to avoid the addition of large volumes of spent medium and consequent dilution of medium because of large volumes of viral stock for high cell density infection. The volume of virus stock added should not exceed 2% of the total culture volume.

1. Ultracentrifuge virus stock at 48,000g for 2 h at 4°C (Beckman, L-8 Ultracentrifuge, Beckman Inc.).
2. Discard supernatant and resuspend the pellet in fresh serum-free medium and sterilize by filtration through a low protein-binding 0.22- $\mu$ m membrane (Corning Inc.) (see **Note 10**).
3. Alternatively, virus stock concentration can also be accomplished by using ultrafiltration with a 50,000-molecular weight cutoff membrane followed by a diafiltration step (see **Note 11**).

### 3.2.3. Baculovirus Quantification

Several methods are used by various researchers in the field to determine the virus titer (*see* Chapters 4, 5, 10, and 21 for alternatives to that given here). These include antibody-based assays (**9,10**) and quantitative real time PCR methods using baculovirus genome sequences (**11**). The plaque assay determines the number of virus particles that form a plaque on a lawn of insect cells following baculovirus infection and, thus determine the number of infectious particles in a given baculovirus preparation. The end point dilution assay is also a measure of infectious virions in a viral stock.

It should be emphasized, regardless of the method used to quantify the virus titer, that it is important to carry out a few small scale shake flasks experiments to verify the quantity of virus stock needed to achieve growth cessation, which is indicative of a synchronous infection. The production of the recombinant protein should also be determined prior to any scale up in the bioreactor (*see* **Note 12**).

The method described herein has been extensively used in our laboratory. It is based on flow cytometry measurements of labeled virus particles and measures the total virus particle concentration (**12**). Specifically, it is based on the binding of a fluorescent dye (SYBR green) 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 FACS. 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. This method, however, cannot give direct information on the infectious particles. It is possible to correlate the total particle number obtained by this method to the pfu per milliliter obtained using either a plaque assay or the end point dilution assay. The procedure is briefly described next and additional details can be found elsewhere (**12**).

1. Dilute the baculovirus stock solution with PBS buffer (without calcium and magnesium) to  $10^{-3}$  or  $10^{-2}$ . These dilutions should be sufficient for titers of  $10^7$  to  $10^9$  virus particles per milliliter.
2. Add 850  $\mu$ L PBS buffer and 100  $\mu$ L of the diluted virus solution to a screw-capped 1.5-mL microtube (Starstedt).
3. Add 20  $\mu$ L 5% paraformaldehyde (in PBS) to the tube and maintain at 4°C for 1 h to complete fixation of the viral particles.
4. Freeze the fixed sample in either a mixture of dry ice and ethanol or liquid nitrogen for at least 10 min before thawing the sample in a water bath at room temperature.

5. Add 10  $\mu\text{L}$  10% Triton X-100 (in PBS) to the thawed sample and allow to set at room temperature for 5 min to permeabilize the baculovirus particles.
6. Dilute SYBR Green I stock solution 200 times.
7. Add 20  $\mu\text{L}$  diluted SYBR Green to stain the sample.
8. Heat the stained sample in a water bath in the dark at 80°C for 10 min.
9. Cool the sample on ice and transfer to 5-mL disposable polystyrene tubes.
10. Calibrate the sample flow rate in the flow cytometer to count approx 100–500 events per second.
11. Determine the viral particle count using the FACS (EPICS XL-MCL) by counting each sample for 30 s.
12. Calibrate the particle counts per milliliter using a standard fluorosphere solutions ( $C_F$ ).
13. Calculate the virus particle concentration using **Eq. 4**:

$$\frac{\text{Virus particles}}{\text{mL}} = \frac{C_V \times D \times 5 \times 10^7}{C_F \times V} \quad (4)$$

where  $C_V$  = particle counts for virus particles,  $C_F$  = particle counts for flowset,  $D$  = dilution factor of the virus solution,  $V$  = volume ( $\mu\text{L}$ ) of the diluted solution used in the sample preparation.

The “ $5 \times 10^7$ ” is obtained by multiplying the final volume (1000  $\mu\text{L}$ ) of sample by the particle concentration ( $5 \times 10^4$ ) of the diluted standard fluorosphere solutions.

### 3.3. Online Monitoring and Control of the Bioreactor Cultures

The online monitoring and control of the DO and pH in the bioreactor are described next.

#### 3.3.1. Dissolved Oxygen

1. Maintain DO at 40% of air saturation using a monitoring and control system as described, for example, in Kamen et al. (13). The control system relies on oxygen supplementation through the use of a mixture of  $\text{N}_2$  or air and  $\text{O}_2$ .
2. Head space aeration is generally sufficient to maintain the DO at 40% in routine batch cultures where the production is carried out by infecting cells at about  $2\text{--}3 \times 10^6$  cells/mL.
3. Overlay flow rate depends on head space volume in the bioreactor, but is typically set at approx 10% the vessel volume per min; e.g., for a 3.5-L bioreactor with a 3-L operating volume an inlet gas flow of 300 mL/min is used to flush the gas phase in the bioreactor head space.
4. Direct sparging is needed to supply adequate oxygen at cell densities greater than  $4 \times 10^6$  cells/mL (see **Note 13**).

#### 3.3.2. pH

The pH is monitored, but control is usually not required for Sf-9 insect cells (see **Note 14**).

### 3.4. Advanced Online Monitoring

The advanced monitoring systems in use in our laboratory for monitoring the carbon dioxide evolution rate (CER) and the biomass using capacitance measurement and its role in the development of robust processes is discussed in this section.

#### 3.4.1. Carbon Dioxide Evolution Rate

Insect cells do not require CO<sub>2</sub> in the gas mixture. However, the measurement of CO<sub>2</sub> in the bioreactor exhaust gas can be used to quantify the cell respiration rate and thus characterize the physiological status of the cells. CO<sub>2</sub> can be measured online by passing the vent gas through an infrared analyzer (Servomex 1400). In insect cells it has been reported that following infection there is a surge in the metabolism and that this is reflected by a peak in the CER at 18–24 h pi (13). 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. Delayed peak of CER indicates that infectivity was insufficient and a nonsynchronous infection has occurred.

#### 3.4.2. Biomass Monitor

The biomass monitor uses capacitance measurement to monitor the biomass. The details of measuring the viable biomass and the biovolume using the capacitance measurements have been described in earlier work from the authors' laboratory (14,15).

The principle of measurement 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 integrity of the cell membrane. Thus, dead cells do not act as a capacitor because they cannot maintain their membrane integrity. Thus, this measurement relies on the number of viable cells in the culture. The profile of the capacitance follows the cell (viable) 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. Further, it can be seen that once the cell diameter reaches a maximum value, there is no further increase in the capacitance value, subsequently showing 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 earlier (14) as well as for high cell densities (6,15) (see also Note 15).

### 3.5. Large-Scale Recombinant Protein Production in Bioreactors

Protein production using insect cells-BEVS can be done in a simple batch mode or at high cell density cultures obtained through the fed-batch culture method.

#### 3.5.1. Bioreactor Sterilization

1. Clean the vessel and rinse well to remove all traces of detergent.
2. Check all O-rings to look for wear and tear; replace as required.
3. Assemble the reactor following instructions in the operation manual.
4. All accessories, e.g., bottles for addition of inoculum, acid, base, nutrient supplementation, and so on, 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 hoods. All sterile transfer is to be done in the hood. It is preferable to locate the bioreactor as close to a biological hood as possible.
5. The pH probe should be calibrated prior to sterilization.
6. The membrane and electrolyte of the 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.
8. The sterility of the bioreactor can be verified prior to addition of cells by leaving the medium without cells overnight at 27°C.

#### 3.5.2. Production of Protein in a Simple Batch Culture

This is the most commonly used method for operating insect cell-BEVS processes.

1. Inoculate the bioreactor at a cell density of approx  $0.5 \times 10^6$  cells/mL by combining cells from exponential growth phase cultures (see Chapter 1) 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 (see Note 16).
3. Cells are grown to a density of about  $2.5\text{--}3 \times 10^6$  cells/mL when they are infected by the addition of baculovirus stock.
4. Synchronous infection may be achieved by using a MOI greater than approximately five (see Note 12). Because the methods of titration used result in a wide

variation of baculovirus stock titers it is common to use an MOI of approx 10 to obtain a synchronous infection. The volume of baculovirus stock is determined according to **Eq. 5**.

$$\left\{ \begin{array}{l} \text{Volume of baculovirus} \\ \text{stock to add to bioreactor (mL)} \end{array} \right\} = \frac{X \times V \times \text{MOI} \times 10^3}{P} \quad (5)$$

where where  $X$  = viable cell density (cells/mL),  $V$  = bioreactor volume (L), MOI = target multiplicity of infection (pfu/mL) and  $P$  = baculovirus stock titer (pfu/mL).

Typical profiles of recombinant protein production using the bioreactor conditions and monitoring systems previously described are shown in **Fig. 2**. From these results it can be seen that the profiles of the capacitance follow closely the viable cell density in the growth phase. A linear relationship can be established for the capacitance during the exponential growth phase. Following infection the capacitance profile follows the increase in diameter of cells. The profile shows a plateau at approx 24 h pi. This time may vary depending on the synchronicity of the infection. The CER profile shows an increase during the growth period corresponding with the increase in the number of cells. A peak in the CER is observed at about 18–24 h pi is concomitant with the plateau in the capacitance profile. The CER and capacitance profiles provide an accurate picture of infection progress. There is a decrease in the capacitance that corresponds to the loss of cell viability in the later period of the infection (48–72 h pi). This is accompanied by a decrease in the CER. Thus, online monitoring can be used to determine the time of harvest. In case of asynchronous infection conditions, the plateau in the capacitance and the peak in CER occur at a time later than 24 h pi. SEAP production, as quantified by enzyme activity, commenced at 24 h pi and continued increasing until about 72 h pi. The viability at this time rapidly declined; therefore, the culture was harvested at this time.

### 3.5.3. High Cell Density Culture Using Fed-Batch Method

Previous work in the authors' laboratory showed that a nutrient feed comprised of a full complement of amino acids, yeastolate, vitamins, and trace elements (**16–18**) could yield a 1.4- to 4-fold increase in  $\beta$ -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 online 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. Recombinant protein production is now done routinely at cell densities of  $10^7$  cells/mL.

1. The nutrient concentrate comprises glucose, amino acids, yeastolate, lipids, vitamins, and trace elements.

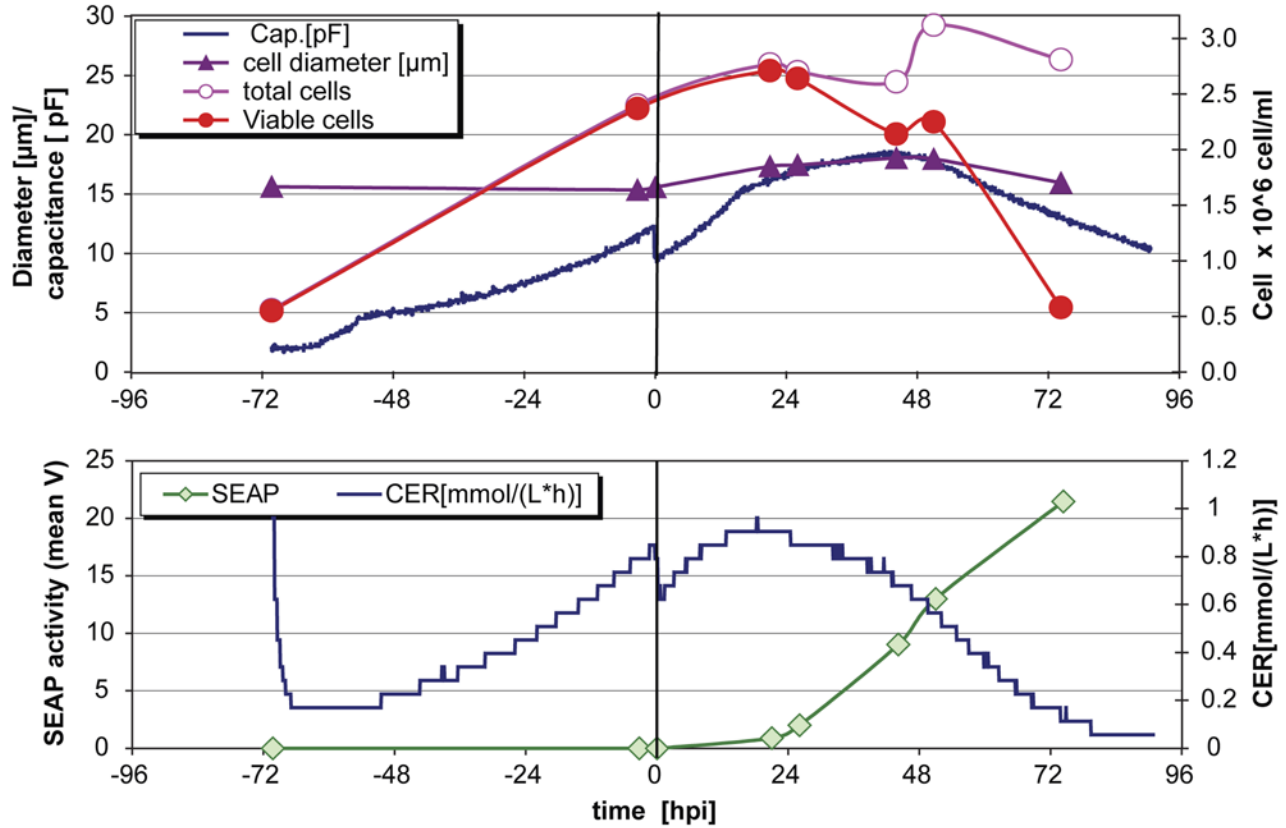


Fig. 2. Production of recombinant secreted alkaline phosphatase (SEAP) in the Sf-9/BEVS system in a batch stirred tank bioreactor under controlled conditions with online monitoring of the biomass by capacitance and the carbon dioxide evolution rate (CER).

2. The details of the components of the amino acid and lipid mixtures have been described earlier in Bédard et al. (15). The feed concentrates can be prepared prior to use, sterilized by filtration, and stored frozen at  $-20^{\circ}\text{C}$ .
3. Inoculate the bioreactor at cell densities of approx  $0.5 \times 10^6$  cells/mL from exponential growth phase cultures (see Chapter 1).
4. The volume of one feed addition is approx 11.0% v/v of the culture volume.
5. Select a rate of feeding such that one addition is made over a period of 24 h. This is done mainly for technical reasons and can be changed depending on the schedule in the users' laboratory.
6. The volume in the bioreactor may be kept constant by removing volume between two additions of nutrient feed.
7. The culture is infected when the cell density reaches  $10^7$  cells/mL.

A typical profile of recombinant protein production at high cell density is shown in Fig. 3A,B. The cells continued to grow exponentially and reached a density of  $10^7$  cells/mL. At this stage the cells in the bioreactor were infected with the virus at a MOI of one. A second feed of nutrients was added at the time of infection and a third at 24 h pi. There was a period of slow growth during the period following the initial feed that was because of a technical problem with the oxygen supply. However, once the problem was rectified the cells resumed their exponential growth and reached a density of  $10^7$  cells/mL, at which time they were infected with the virus. The cells were infected synchronously as can be seen from the profiles of total and viable cell density where there was no further increase in cell number. The cell size continued to increase accompanied by an increase in the capacitance profile. The cell diameter showed an increase up to 72 h pi followed by a decrease. This decrease was concomitant with decreases in the viable cell density and the capacitance (Fig. 3A). The peak in CER was at approx 24–30 h pi. Recombinant protein production, as measured by an ELISA assay, was detected in the supernatant at 48 h pi and continued to increase until 72 h pi. The peak in production occurred between 60 and 72 h pi, after which there was a decrease in the intracellular protein measured. This decrease followed the decline in viable cell density and therefore indicated the optimum harvest time. A fourfold increase in the volumetric yield of protein was observed when compared to the production obtained when cells were infected at a density of  $2.5 \times 10^6$  cells/mL (Fig. 3B).

A summary of our work involving the production of different proteins using the high cell density method is given in Table 1. It can be seen from this table that this method is robust, i.e., it can be used for different proteins, e.g., galactosidase  $\beta$ -(gal) or HSVrgD2 membrane receptor, and can be done using different serum-free media formulations. It has also been successfully adapted to Sf-21 cells and used for the production of complex proteins using co-infection processes, e.g., cytochromes P450s (CYP 3A4 and CYP 2A1).

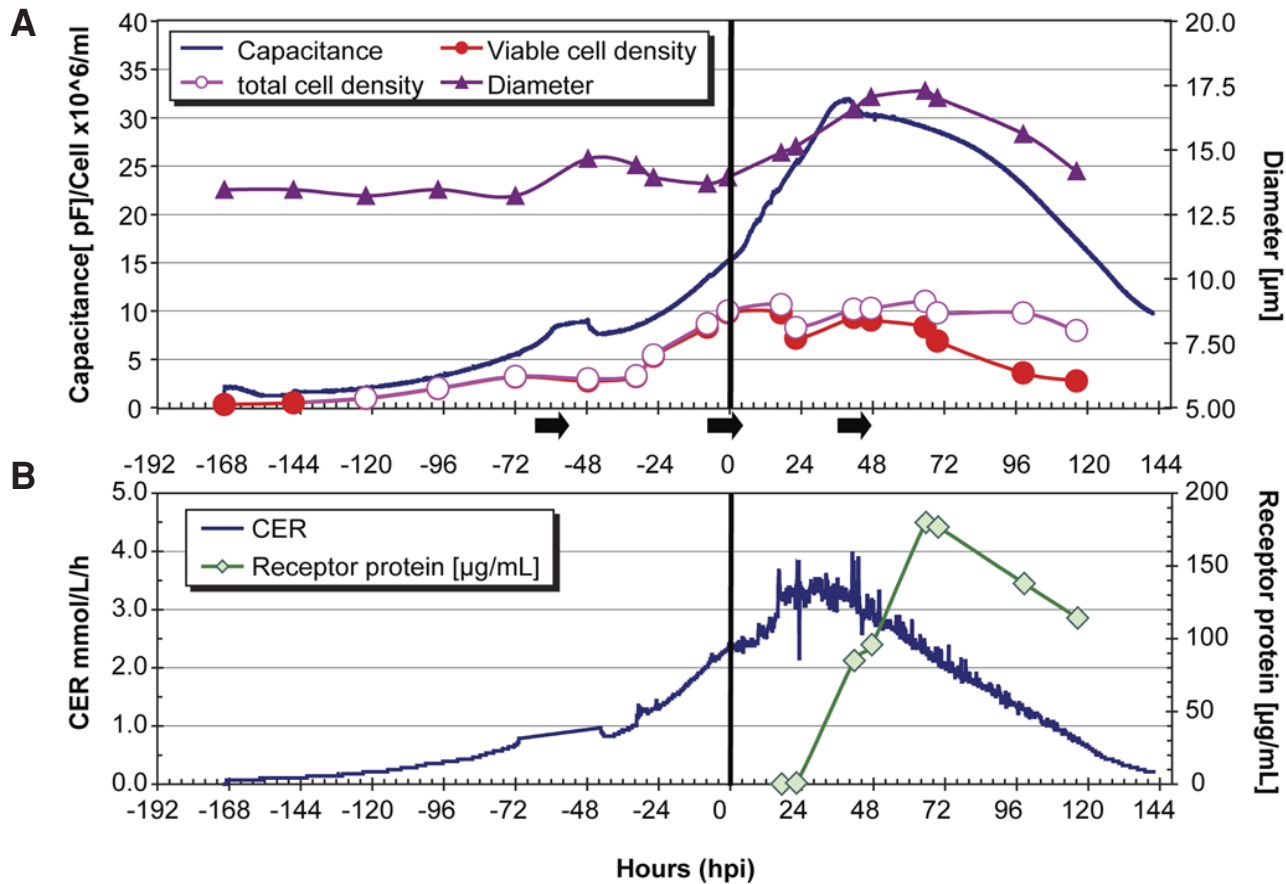


Fig. 3. Profiles of capacitance and carbon dioxide evolution rate (CER) from online monitoring and total and viable cell density, cell diameter and receptor protein concentration from offline analyses of a fed-batch culture using Sf-9 cells infected at  $10^7$  cells/mL.

**Table 1**  
**Summary of Fed Batch Experiments Carried Out With Different Cell Lines**  
**and Media**

Cell line	Product	Medium	Cell density at infection in batch 3 $10^{-6}$ cells/mL	Cell density at infection in fed batch 3 $10^{-6}$ cells/mL	Volumetric yield in Batch	Volumetric yield in fed batch
Sf-9	$\beta$ -gal	SF-900II	2.5–3	13	77.5 IU/mL	372.2 IU/mL
Sf-9	HSVrgD2	Excel-420	3	10	36.8 mg/mL	180 mg/mL
Sf-9	HSVrgD2	IPL-41	2.5	10	48.9 mg/mL	200 mg/mL
Sf-21	CYP 3A4	Excel-420	2.2	6.3	26.4 nmol	40.5 nmol
Sf-21	CYP 2A1	Excel-420	2.5	6.0	10.3 nmol	52 nmol

### 3.5.4. Harvesting the Bioreactor and Primary Separation for Product Recovery

The time of harvest is determined by the MOI used and the production profile determined by small-scale testing.

The bioreactor should be harvested before there is a significant decrease in the viability of the culture. Normally, the culture is harvested when the culture viability is between 70 and 75%. This may, however, be dependant on the type of protein. The typical procedure is given next.

1. Connect a sterile bottle to the harvest line of the bioreactor.
2. Lower the temperature set-point of the bioreactor to about 25°C.
3. Decrease the agitation speed to about 60–70 rpm.
4. Collect the cell culture suspension by opening the harvest valve.
5. The remaining operations may be done at 4°C, depending on protein stability.
6. Centrifuge the cell suspension at 1000g if the product is intracellular and at 1500g if the product is secreted and is in the supernatant.
7. Separate the cells and supernatant. Store at –80°C until further processing.
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.

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.

## 4. Notes

1. There are several serum-free media that are commercially available that are suitable for insect cell lines, including the Excel series of medium from SAFC-JRH Biosciences (St. Louis, MO), ESF-21 (Expression Systems, Woodland, CA), HyQ® SFX-Insect™ (Hyclone, Logan, UT), Insectagro™ (Krackelar Scientific, Albany, NY), Insect-XPRESS™ (Cambrex- Biowittaker Biosciences, Walkersville, MD), and BD BaculoGold Max-XP (BD Biosciences, Franklin Lakes, NJ). The list is representative and not exhaustive, new serum-free media become commercially available periodically. A more extensive list of commercially available serum-free media and details about developing your own serum-free medium are discussed in Chapter 8.
2. The problem of viral 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 10% fetal bovine serum. Additional details can be found in Chapter 9.

3. These tubes must be used to avoid liquid loss during the heating process.
4. It should be noted that care should be taken when using a gas mixing systems designed for mammalian cell cultures that employs CO<sub>2</sub>, which may result in pH changes during the culture. Insect cells do not require CO<sub>2</sub> 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<sub>2</sub> altogether by replacing it with N<sub>2</sub> or air.
5. A Coulter counter (Beckman Coulter, Inc., Fullerton, CA) may be used to determine the total cell concentration and the average cell diameter. It is a quick method, but lacks the ability to determine cell viability. Other automatic devices are commercially available that performs cell viability, cell number, and cell diameter. For example, the CEDEX from Innovatis (Bielefeld, Germany) and the ViCell from Beckman Coulter, Inc.
6. The flasks should be cleaned thoroughly to remove traces of detergent prior to steam sterilization.
7. The measurement of the biomass offline using a Coulter Counter (which measures cell size and total number), as well as the traditional Trypan blue exclusion method for cell viability, help to paint an accurate picture of the physiological conditions in the bioreactor.
8. The MOI is defined as the ratio of 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.
9. 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. The cell diameter should begin increasing by 24–48 h pi. Harvest should only take place following the concomitant decrease in cell viability and the increase in cell diameter.
10. The filtration of the freshly made virus stock ensures the sterility and removes cell debris. The filtration step must be done immediately because of the possibility of a dramatic decrease in titer following virus stocks settling over 1-wk period.
11. The 5-ft<sup>2</sup> Pellicon tangential flow filtration unit (Millipore Corporation, Billerica, MA) can be used for larger volumes and smaller volumes can be concentrated using a 0.75-ft<sup>2</sup> minisette Filtron unit (Pall Corporation, East Hills, NY). These protocols also require a peristaltic pump and a reservoir. An additional diafiltration step is needed to exchange the spent medium in the concentrate with fresh medium before use. It has been found that such concentrated stock can be stored in fresh serum-free media without significant loss of virus titer at 4°C for a period up to 6 mo.
12. The MOI is defined as the ratio of pfu per cell at the time of infection. At a given time the probability of a cell being infected can be described by a Poisson distribution (19). See Chapter 1 for more details about the Poisson distribution. According to the Poisson distribution, the proportion of uninfected cells in a

population is given by  $e^{-\text{MOI}}$ ; thus, an MOI of one will result in 36% of the cells remaining uninfected (20). Cells are also known to be infected best when in exponential growth phase and thus the distribution of the cell population in different phases of growth may additionally play a role in achieving a complete infection of the population. Thus, it is essential to determine in shake flask experiments the MOI required to stop growth at 24 h pi to verify synchronous infection (if desired). At lower MOI, one may rely on secondary infection to complete the infection process; as a consequence the production process is delayed.

13. In some bioreactor configurations a minimal sparging flow rate of an O<sub>2</sub>-enriched gas mixture is used to minimize the shear-stress associated with direct sparging. This is done using a small capacity sparger (diameter = 1 cm, length = 2 cm) made of polypropylene with an 80- $\mu\text{m}$  pore size (Porex Technologies, Fairburn, GA). For example, in a 3-L bioreactor (operating volume), 10 mL/min of the total inlet gas flow of 300 mL/min is sparged into the culture, with the remainder used to flush the bioreactor head space. Alternatively, sparging of pure O<sub>2</sub> might be used in a pulse mode with a minimal flow rate.
14. Sf-9 cells do not normally produce significant amounts of either lactate or ammonia as metabolic byproducts (21), 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 or lack of oxygen, which results in lactate production as a result of glucose metabolism under oxygen starvation conditions. In contrast, Tn-5 cells used at high cell densities can result in significant pH change because of byproduct accumulation. However, pH control of Tn-5 cultures is usually unnecessary because most of the corresponding pH change only occurs upon cell death. An exception would be if the recombinant protein was particularly pH sensitive.
15. The combination of the CER profile and the capacitance profile from the biomass monitor can help in designing a strategy for feeding cells to achieve higher densities and also in making decisions on the time of harvest based on the synchronicity of the infection (13). The peak in the CER was shown to occur between 18 and 24 h pi in cultures infected at a MOI resulting in synchronous infection. This is accompanied by a shoulder in the profile of the permittivity that shows a plateau, thereby indicating synchronous infection. In cases where the MOI used is low (i.e., an asynchronous infection) this peak in CER shifts to a later time point and the profile of the permittivity continues to increase, thereby showing postinfection growth. It should be noted that following infection there is a competition for available nutrient resources between virus replication, protein production, and cell growth and maintenance. Thus, postinfection cell growth observed by the online signals of CER and permittivity indicates that additional nutrients may be required. Hence, offline monitoring of glucose or glutamine should be done to ensure that the feeding strategy eliminates nutrient limitations (15).
16. Care must be taken to use cells from the exponential growth phase, whereas using a baculovirus inoculum that does not exceed 10% of the working volume and obtaining the desired starting cell density.

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## Production of Baculovirus-Expressed Recombinant Proteins in Wave Bioreactors

Sue H. Kadwell and Philip I. Hardwicke

### Summary

Wave Bioreactors are relatively new to the field of protein production from insect cells infected with recombinant baculoviruses. Various sizes are available to support expression needs from small to large scale. They offer many advantages over stirred tank and airlift reactors, including simple operation, ease of setup and clean-up, and minimal utility requirements. The design consists of a platform rocker onto which presterilized and assembled bags are placed. Once inflated, the bags are filled with medium and cells. Filtered air flow maintains the volumetric shape of the bag and provides head space for gas exchange. A “wave” motion is created by the rocking and angle settings of the platform. Monitoring can be as simple or as detailed as the user requires. Excellent insect cell growth and production of proteins from many classes can be achieved with these units.

**Key Words:** Wave bioreactor; “cellbag” or “wavebag”; Sf-9 cells; *Trichoplusia ni* cells; recombinant baculovirus; mass spectrometer; cellbag integrity testing.

### 1. Introduction

At the time of the introduction of Wave Bioreactors in 1999 (*1*), options available for suspension cell cultivation included shake and spinner flasks, roller bottles, stirred tank, and airlift bioreactors. Cultivation was somewhat limited in scale because of gas transfer mechanisms, as shear and bubble burst effects within the vessels were injurious to cells. Modifications to stirred tank or airlift bioreactors and medium additives, e.g., Pluronic® F-68, were identified to alleviate these effects. The wave bioreactor concept was introduced as an alternative bioreactor option for suspension cell culture without the need for protective medium additives.

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

For many years, laboratories used tilting or rocking platforms to mix reagents. The basic premise of the wave bioreactor is a rocking platform, i.e., a “wave machine,” onto which a sterile bag is placed. This is a plastic “bioreactor,” using conventional biological fluid handling technology specially adapted for this purpose. Several systems currently exist, from 1 to 1000 L chambers, with 100 mL to 500 L working volumes. The “cellbag” or “wavebag” is inflated with gas (filtered air, CO<sub>2</sub>, or O<sub>2</sub> enriched air). A wave is generated by using a simple reciprocal rocking motion. Gas transfer is very efficient with a large gas–liquid interface, and it is in this regard that wave machines work well, even at large scale.

Wave bioreactors score very high in reducing manpower requirements because the bags are presterilized ( $\gamma$ -irradiated), precluding sterilization and cleaning cycles. This feature greatly reduces contamination risks. Even at the largest scale, bags can be harvested and a fresh one restarted within one working day. This is quite difficult for other large-scale systems.

The wave systems are simple, which aids training and reduces costs. For a culture requiring air alone, such as the baculovirus expression vector system (2), the only facilities requirement is electricity. The air can be pumped from the room directly by the machine itself. The units can be purchased with heater plates to maintain culture temperatures, or the bioreactors can be set up and run in a temperature-controlled cabinet or room. Since the original machines were developed, additional techniques have been utilized, such as weldable tubing (e.g., C-Flex or Pharmed) for sterile additions. Tube sealers and simple blood collection Vacuette tube sampling systems are also used, which help to maintain sterility (these are also useful for containment when working with higher risk organisms). Also, wave cellbags can be integrity tested by using flow-controlled process air supplies.

Although the systems are simple, they are not restrictively so. Dissolved oxygen and pH probes can be fitted, sample lines can be connected to automated biochemical analyzers, and the exit gas can be analyzed using mass spectrometry. As this suggests, the wave bioreactor concept is a very powerful tool in cell culture, particularly as the amount of biological materials needed often requires large cultures to be grown efficiently.

Wave bioreactors have been used to grow many cell culture types. Suspension cultures of animal (NS0, CHO, HEK, HL60, hybridoma), insect (Sf-9, Sf-21, and Tn-5) and plant cells have all been grown successfully (3). Microcarrier cultures have also been grown, as have perfusion cultures. Perfusion cultures rely on a specialized wave cellbag fitted with a cell filter (4), which enables cell-free material to be removed and fresh medium added to the culture. It has been reported with this method that monoclonal antibody yields have been



Fig. 1. Photograph of Wave System200EH with inflated Cellbag200 set up in GSK RTP fermentation facility. In the background, System20s and System50s can be seen.

boosted significantly (5). It is probable that wave cellbags could also be interfaced with more complex control systems, allowing automated set point alteration and feeding regimes to be implemented. In this way, the wave bioreactor could become an even more powerful ally for cell culture production methods.

Our chapter focuses on the use of wave bioreactors with Sf-9 and Tn-5 cells at the 10-, 25-, 50-, and 100-L scale (Fig. 1). For our laboratory, the positive benefits of the wave bioreactors have outweighed any negative points (Table 1). We have produced many different classes of recombinant proteins successfully in the wave bioreactors, including kinases, secreted proteins, membrane proteins (e.g., G protein-coupled receptors and ion channels), nuclear receptors, and others.

## 2. Materials

1. The Wave Bioreactor System20/50 can be purchased in the United States from Wave Biotech (<http://www.wavebiotech.com/>), and similar units, BioWave20 SPS and BioWave50 SPS, can be purchased from Wave Biotech AG in the United Kingdom (<http://www.wavebiotech.ch/>). Along with the basic unit, a Heater20/50 temperature control, LoadCell20 Weight Indicator, O2MIX20/air controller, and a DO20 dissolved oxygen monitor can also be purchased.

**Table 1**  
**Advantages and Disadvantages of Wave Bioreactors**

	Advantages	Disadvantages
House system requirements	No steam requirements, only electricity required. Any additional gas (CO <sub>2</sub> , for example) can be from tanks.	
Bioreactor setup	Can easily be assembled/set up by lab personnel.	
Media fill, cell addition, and virus addition	Easily accomplished with high degree of sterility with tube welder. Without tube welder, can use quick-connect ends, yet sterility may be compromised.	Additional purchase of tube welder, and sealer recommended.
Sampling	Quick and easy. No need to steam across sample connector. If using higher category biologicals, can use vacuette samplers ( <i>see Subheading 2., item 19</i> ).	
Harvest	Similar to other bioreactors.	
Clean-up	Autoclave used bag, and discard.	
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 set up, cleaning labor.

2. The Wave Bioreactor System200EH can be purchased from Wave Biotech in the United States. The BioWave200 SPS was purchased from Wave Biotech AG in the United Kingdom. Along with the basic unit, an optional Loadcell200 Weight Indicator and a DO200 dissolved oxygen monitor can also be purchased.
3. Wave Cellbags (Wave Biotech).
4. Sterile Tube Fuser (<http://www.wavebiotech.com/>) or Reewelder (<http://www.wavebiotech.ch/>) with interchangeable tubing holders can be obtained for tube welding aseptically outside of a biological safety cabinet.
5. Terumo Sterile Connecting Device (SCDIIB, <http://www.terumo.com/>) can be used with 1/8" ID, 1/4" OD C-flex tubing.

6. Hot Lips or Ree Sealer tube sealer can be obtained from Wave Biotech for thermal sealing of fused/cut tubing.
7. Nova Bioprofile 400 (Nova Biomedical Corp., <http://www.novabiomedical.com>) for monitoring the culture medium.
8. Guava PCA (Guava Technologies, <http://www.guavatechnologies.com/>). Vialflex reagent (Guava Technologies). Also used for cell counting is a ViCell XR, from Beckman Coulter (<http://www.beckmancoulter.com/>), which is an automated Trypan blue exclusion method.
9. CARR® ViaFuge® Pilot 9004 (<http://www.pneumaticscales.com/>).
10. Harvest rigging set (custom order from BIOEAZE™, SAFC Biosciences, <http://www.safcbiosciences.com/>).
11. Mass Spectrometer, Prima DB (Thermo Onix, <http://www.thermo.com/>) for off gas analysis.
12. *Spodoptera frugiperda* (Sf-9) cells can be obtained from American Type Culture Collection, Rockville, MD. Sf-9 cells can be maintained in any IPL-41 formulation; our line was adapted in-house to a GlaxoSmithKline (GSK) formulation of IPL-41 and maintained at viabilities greater than 97%.
13. *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5, T.ni) cells (commercially known as High Five™) can be obtained from Invitrogen (Carlsbad, CA). Cells were maintained in EX-CELL™ 405 (SAFC Biosciences) at viabilities greater than 97%.
14. GSK-IPL-41 cell culture medium was prepared by SAFC Biosciences, from a GSK specified formulation. Medium can be received in custom bags to specifications for welding to cellbags.
15. EX-CELL 405 cell culture medium was purchased from SAFC Biosciences in custom bags to customer specifications for welding to Cellbags.
16. Infors Multitron II Orbital Shaking Incubator was purchased from Appropriate Technical Resources (<http://www.atrbiotech.com/>).
17. For cell culture, various sizes of polycarbonate disposable flasks, with vented caps, were obtained from Corning. Cells for inoculum are grown in 2- or 3-L Corning polycarbonate disposable flasks with vented caps (Corning, <http://www.corning.com/>).
18. Palltronic Filter Integrity Tester (Pall Corporation, <http://www.pall.com/>).
19. For sampling bioreactor: sterile alcohol pads and 10-cc sterile syringes can be obtained from various suppliers. For higher assurance of sterility or containment purposes, a Vacuette system can be used (MacoPharma Phlebotomy kit [VSL7000YQ]), available in the US from United Pharma, GA, for non-medical use only. Vacuette tubes, obtained from Greiner Bio-One, using either 6 or 9 mL capacity (part numbers 456001 or 455001, respectively, or 456016 [for US 6-mL tubes]).
20. For cell and virus addition, custom bags from TC Tech (<http://www.tc-tech.com>) can be used. The bags are presterilized and preassembled in a range of sizes (300 mL, 2 L, and 5 L) with a central port for liquid addition and C-flex tubing for welding to wave cellbags. A custom stainless steel stand for supporting the bags during liquid addition inside a biological safety cabinet was designed and produced by STI (<http://www.stiflow.com/>).

### 3. Methods

#### 3.1. Preparation of Inoculum

1. Sf-9 cells are grown in GSK-IPL-41 medium at 27.5°C with shaking at 110 rpm in an Infors incubator. Disposable polycarbonate Corning Erlenmeyer flasks with vented lids are used. Cells are routinely passaged three times per week, seeding at  $0.3 \times 10^6$  cells/mL and splitting when cells reach approx  $2\text{--}4 \times 10^6$  cells/mL. Cells are transferred to new flasks when there is a ring buildup at the liquid interface. Cells are counted using a Guava PCA and Viaflex reagent diluted into GSK-IPL-41, or a ViCell Cell Viability analyzer. Alternatively, as discussed elsewhere in this book, (1) a hemacytometer can be used to determine the cell density and viability (*see* Chapter 11), (2) other methods of growing cells to inoculate the wave bioreactor can also be used (*see* Chapter 9), (3) other cell growth media can be used (*see* Chapter 8), and (4) other cell lines can be used (Sf-9 cells are used as an example herein) (*see* Chapter 6).
2. For the inoculum, Sf-9 cells are grown in GSK-IPL-41 in disposable polycarbonate Corning flasks with vented caps. We use up to 1 L in the 2-L flask and 2 L in the 3-L flask with excellent growth and viabilities. Viabilities should be greater than 97%.
3. Tn-5 cells are grown in EX-CELL 405 at 27.5°C with shaking at 110 rpm in an Infors incubator. Disposable Corning Erlenmeyer flasks with vented lids are also used. As with our Sf-9 line, cells are routinely passaged three times per week, seeding at  $0.3 \times 10^6$  cells/mL and splitting when cells reach approx  $2\text{--}3.5 \times 10^6$  cells/mL. Cells are transferred to new flasks when there is a ring buildup at the liquid interface. Cells are also counted with a Guava PCA, using Viaflex reagent diluted in EX-CELL 405, or a ViCell Cell Viability analyzer. We try to prevent this line from reaching a density more than  $4 \times 10^6$  cells/mL, as the viabilities can be greatly affected.
4. For the inoculum, Tn-5 cells are grown in EX-CELL 405 in 2- or 3-L disposable Corning flasks with vented caps. The maximum volume is limited to 750 mL in the 2-L or 1.5 L in the 3-L flask as this cell line has a higher oxygen requirement than the Sf-9 cells. Viabilities should be greater than 97%.

#### 3.2. Setup of the Wave Cellbag

The rocking platform on the Wave units is cleaned with 70% Isopropanol. For the Wave System200, the inside of lid is also wiped down. (Please note: for rapidly stopping the unit, the large red Emergency Stop button on the front panel of the Wave 200EH units should be used.)

The cellbag name denotes the entire volumetric size of the bag, not the volume that can be used for a run. The maximum level the bags can be filled is 1/2 the volumetric amount (e.g., a Cellbag50 can only be filled with media 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 for using different sizes of wave cellbags. 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 the bioreactors are not in a temperature-controlled cabinet or room (*see Note 1*).

There are at least two filters provided on all the 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. A heater jacket is used around the exhaust filter to decrease condensation build up. The third filter on the Cellbag100 and 200 sizes is a spare filter to be used if the exhaust filter becomes clogged. This spare filter should be clamped off unless needed.

On the Cellbag20s and 50s, a restrictor is added to the exhaust filter by the manufacturer to keep the bag taut after inflation. For the European version of the System 200, a restrictor is also placed downstream of the vent filter to maintain the bag pressure (*see Note 2*).

### 3.2.1. Setup of Wave System200

1. Placement of the Cellbag: turn cams inside the wave lid counterclockwise to open. Slide the edge of cell bag with the fiberglass rods into the holder 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 most filter on the Cellbag. On the control panel, the in port should be left empty. If using two Cellbag100s, 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, place a heater jacket on the exhaust filters on each bag.
4. Open both the inlet and exhaust filter clamps.
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, and 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. If using a polarographic dissolved oxygen (DO) probe from Wave Biotech (*see Note 3*), check that the DO sensor reading on the control panel is close to zero before the probe is connected. While the bag is inflating, keep probes in their protective sheaths and connect the DO and temperature compensation probes to the panel. Allow 30 min for the DO probe to polarize. After 30 min, insert the

temperature compensation and DO probes by removing from the sheaths and placing into the y fitting (Oxywell port). This is color coordinated; the DO probe slips into the blue port (longest of the two) and the temperature compensation probe into the green port. Handle the probes gently, threading the DO probe as quickly as possible into the port to prevent drying out. Once inserted into the bag, set the DO probe reading to 100%.

9. Set temperature, rock angle, and rock speed on the main screen. Turn on heaters (L and R) for a Cellbag200 and for two Cellbag100s, or just one heater (L) for a single Cellbag100. Temperature control will begin with rocking.
10. Tare LoadCell.

### 3.2.2. 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, then slide opposite rod in, and tighten cams. Make sure bag is locked in place and free of creases.
2. Attach air line from the air pump out port to the inlet filter (filter without the restrictor) on the Cellbag. Place filter heater jacket over the exhaust filter. Open both inlet and exhaust filter clamps. Inflate the bag by switching on the air pump to 0.45 L/min. When filled, the bag should be tight without creases. Decrease the air flow to 0.25 L/min for a Cellbag20 or to 0.4 L/min for a Cellbag50. Test restrictor if air is not obviously flowing through.
3. If using a DO probe from Wave Biotech, follow the instructions outlined in **Sub-heading 3.2.1., step 8.**
4. Set temperature, rock speed, and angle on platform with control panel.
5. Tare LoadCell.

### 3.2.3. Pressure Testing Cellbags Using the Pall Filter Integrity Tester

For work using higher than basic category organisms, it was necessary to integrity test the Cellbag. Additionally, GSK specified and purchased a number of BioWave20 units from Switzerland (designated System 20-SPS-F), which were fitted with a process air supply line and a pressure sensor on the outlet line. This allowed the units to operate between 8 and 25 mbar. A regulated process air supply (an in-house modification of laboratory air lines) of 60 mbar was provided to the units, while the on-board air pump was disconnected by GSK engineers, with instruction from Wave. In this way, the air supply to the cellbag is controlled and is sufficient to run a Cellbag20. This also allows the use of the Palltronic Filter Integrity Tester apparatus. It can be programmed to supply the bag with air and hold at 75 mbar for 20 min, while any pressure drops are detected. A bag is suitable for use if a pressure drop of less than 5 mbar is observed. Additional safety precautions for higher containment include the use of a blood bag sampling device and Vacuette tubes. A sterile tube fuser is used as in lower category runs, along with a tube sealer to prevent operator exposure when lines are disconnected.

Cellbag200s have been tested, but for our purposes, the larger System200 machines have not been used for higher category work to date.

1. Clamp off all connectors as close to the bag as possible and seat the bag securely in the holder, as described in **Subheading 3.2**.
2. Switch on the Palltronic and allow it to warm up (normally 10 min). It can be supplied with up to eight bar air.
3. Connect the air hose to the cellbag and open the line.
4. Using the menu on the Palltronic, select Forward Flow (FF) and fill in the entries, as required. Most entries are optional. Operator, production area, product batch may be filled in for recording purposes.
5. Proceed to the Test Pressure (mbar) prompt and enter 75 mbar with a 120-s stabilization time and a 20-min hold time.
6. Select 5 mbar as the pressure decay limit and press start.
7. The bag will inflate and then run through the test. If successful, the bag can be run for the experiment. Occasionally, a repeat test may be required if the bag is not seated properly. If a repeat test is required, deflate the bag, and repeat from **step 1**.

### 3.3. Seeding 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. Before cell addition, the appropriate tubing on the inflated cellbag is welded to the tubing on the medium bag with the Sterile Tube Fuser. Prewarmed medium is added by gravity or via a pump. On all cellbag sizes, we use the 1/4" ID, 7/16" OD C-flex line for medium addition. When making additions into the cellbag, the restrictor on the exhaust filter should be removed during the process to prevent high pressure. Monitor the temperature closely, as the temperature may overshoot the set-point (this can be prevented by increasing the set-point stepwise).
2. Using the LoadCell monitor on the Wave units, medium is measured to within 1–2 L of the desired seed volume (1 L = 1 kg).
3. Once addition is complete, the medium line is sealed using the tube sealer and the tubing is cut to detach the media bag. Care is taken to use as little of the C-flex line as necessary, as other additions will be made through this line.
4. The inoculum of cells, at approx  $3 \times 10^6$  cells/mL, is added to the necessary amount of medium in a sterile addition bag (**Fig. 2**) to yield a final cell density of  $0.3 \times 10^6$  cells/mL in the total seed volume. Cells and medium are added into the addition bag within a biological safety cabinet (*see Note 4*).
5. The C-flex line from the bottom port of the addition bag is welded onto the cellbag for cell addition. This is also through the 1/4" ID, 7/16" OD C-flex line.
6. After cells have been transferred into the cellbag by gravity flow, the addition line is sealed using the tube sealer and the bag is cut free.

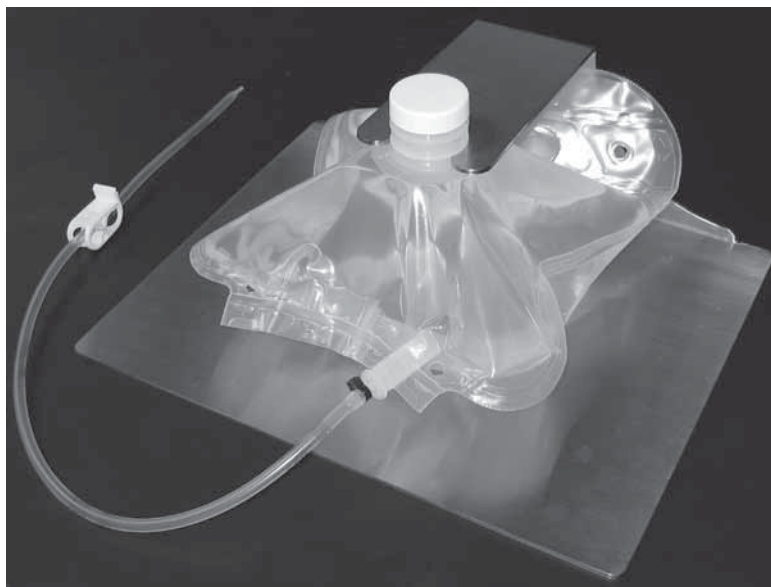


Fig. 2. Two-liter addition bag and holder. Various bag sizes are available and are used for cell and virus addition by welding to Cellbag with Sterile Tube Fuser.

7. **Table 2** lists recommended rock speeds, angles, and air flow. Monitor these settings closely. A clear surface “wave” action should be observed in the bag, without intense force. Monitor for too much foaming, or pockets of foam building up in corners. Adjust rock speed/angle to prevent this (*see Note 5*).
8. Cell number is monitored (**Subheading 3.5.**) and when the density reaches approx  $3 \times 10^6$  cells/mL, medium is added to final production run volume by fusing tubing from the cellbag to medium bag as described in **steps 5** and **6**. For Cellbag100 and 200 sizes, our medium is in custom large bags within barrels, with enough C-flex tubing to allow the use of a pump for medium addition.
9. Rock speed and angle settings are adjusted after medium addition. Suggested settings are shown in **Table 3**. Monitor run daily, and if the DO level drops to less than 50%, adjust rock angle and then the rock speed, checking that an ideal wave is created and that foaming does not become excessive.

### 3.4. Virus Addition

1. Virus stock should be prepared and titered as described elsewhere in this book (*see Chapters 4, 5, 10, 11, and 21*).
2. For protein production runs, virus is added at an MOI of one to five when cells reach approx  $1\text{--}2 \times 10^6$  cells/mL with viabilities greater than 97%. In a biological safety cabinet, the appropriate amount of virus stock is added into a sterile addi-

**Table 2**  
**Suggested Settings for Initial Seed Volumes: Rock Angle and Rock Speed**

Sf-9 cells				
Cellbag	Seed volume	Rock angle	Rock speed (rocks per minute)	Air flow (L/min)
Cellbag20	1–2 L	8.5	24	0.25
Cellbag50	5 L	8	18	0.4
Cellbag100	10 L	6 (5–9) <sup>a</sup>	12(10)	1
Cellbag200	20 L	8	12	2
Tn-5 cells				
Cellbag	Seed volume	Rock angle	Rock speed (rocks per minute)	Air flow (L/min)
Cellbag20	1–2 L	8.5	24	0.25
Cellbag50	5 L	7	15	0.4
Cellbag100	10 L	6 (5–9)	16 (10)	1
Cellbag200	20 L	6 (8)	12	2

<sup>a</sup>( ) Denotes recommendations for Wave Biotech AG Units.

tion bag with a C-flex line attached to the bottom port. The 1/8" ID, 1/4" OD C-flex line on the cellbag is used for virus addition.

3. The C-flex tubing is fused using the Sterile Tube Fuser. Virus is added by gravity flow or by pumping into the cellbag. Pumping will insure complete drainage from the addition line.
4. After addition, the C-Flex line is sealed with the tube sealer, and cut in the middle of the seal to remove the addition vessel.

### 3.5. Monitoring

On all wave cellbags, a CLAVE<sup>®</sup> sampling connector is provided for cell sampling outside a biological safety cabinet. This connector has a luer lock fitting that accommodates a needleless sterile syringe for sampling.

1. Stop the rocking to remove a sample from the cellbag. If using the 200EH 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. It is only necessary to open the Plexiglas window on the 200EH lid.
2. Remove the cap on the sampling connector, and swipe the port with a sterile alcohol pad. Attach a needleless syringe (10 mL) onto the connector. Open the clamp on the tubing of the sampling connector, hold the blue area of the sampling

**Table 3**  
**Suggested Settings for Final Production Volumes: Rock Angle and Rock Speed Settings**

Sf-9 cells				
Cellbag	Final volume	Rock angle	Rock speed (rocks/min)	Air flow (L/min)
Cellbag20	10 L	8.5	24	0.25
Cellbag50	25 L	8	25	0.4
Cellbag100	50 L	6 (7–9) <sup>a</sup>	24 (16, 18, 20)	1
Cellbag200	100 L	8 (8, 9)	18 (18–20)	2
Tn-5 cells				
Cellbag	Final volume	Rock angle	Rock speed (rocks/min)	Air flow (L/min)
Cellbag20	10 L	8.5	24	0.25
Cellbag50	25 L	7	25	0.4
Cellbag100	50 L	6 (7–9)	20 (16, 18, 20)	1
Cellbag200	100 L	7 (8, 9)	20 (18–20)	2

<sup>a</sup> ( ) Denotes recommendations for Wave Biotech AG Units

connector, and draw 10 mL of the sample into the syringe. Close the clamp and remove the syringe. This sample will be discarded because it was used to clear cells from the sampling line.

- Remove the syringe, and swipe 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. Open the clamp on the tubing. Hold the blue area of the sampling connector, draw 10 mL of the sample into the syringe and close the clamp. Remove the syringe. Wipe the port clean, and replace cap to luer fitting on the sampling connector. Turn rocking back on for the unit.
- For higher assurance of maintaining sterility, or if using a higher risk organism, sampling can be done using a Vacuette system. This system uses a MacoPharma Phlebotomy kit. This kit has a luer fitting that is attached to a port on the bag, at the time the bag is prepared, in a biological safety cabinet. The blood bag itself is isolated using the clip, as close to the Y piece as possible. The sheathed needle and housing are then used to sample. When a sample is required, the bag line is opened, the sheath lid is removed and an empty Vacuette tube is inserted. The tube fills with sample, which is discarded as it is used to clear the line. A second tube is then fitted and filled. The line is isolated and the sheath lid replaced after removing the sample tube.

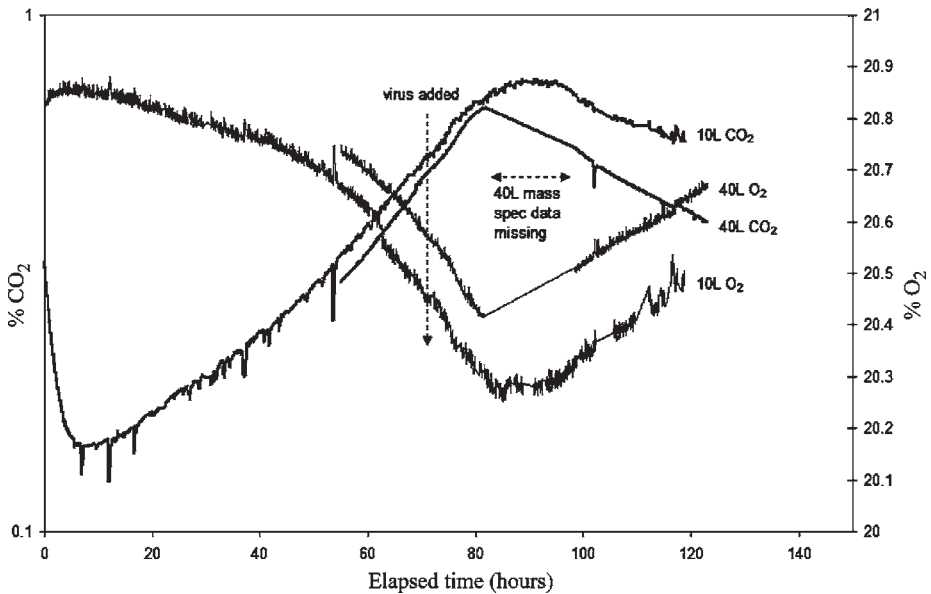


Fig. 3. Mass spectrometer data from BioWave20 and BioWave200 (using 10- and 40-L working volumes, respectively). %O<sub>2</sub> and %CO<sub>2</sub> as measured by Prima DB mass spectrometer, plotted against time of culture growth. As shown in these data, there is a delayed peak in CO<sub>2</sub> evolution and O<sub>2</sub> consumption postinfection.

5. Analyze the sample on a Nova Bioprofile 400. Essential parameters read should include pH, pO<sub>2</sub>, and percent air saturation. Other parameters that can be monitored if desired are glucose, glutamine, glutamate, and lactate. For valid percent air saturation and pO<sub>2</sub> readings on the Nova, samples must be analyzed as quickly as possible or a Nova automatic sampler can be used. If the sample is taken from an EX-CELL 405 production run or other high glutamine-containing formulation, then the glutamine concentration will read off-scale with an undiluted sample. Dilute a second sample 1:10 with PBS and reanalyze.
6. It is also possible to have off-gas analysis performed using a mass spectrometer. Using a Prima DB instrument, cultures can be monitored for O<sub>2</sub> consumption and CO<sub>2</sub> evolution. Monitoring of process air used to supply the bags also allows for concentration differences to be calculated, as is required. In this way, gas concentration time courses can be plotted to check the progress of the culture (Fig. 3). It can be used to generate growth rate information, backing up cell count data.
7. Place approx 2 mL of sample on 60-mm disposable Petri dish to visibly inspect under a microscope.
8. Place 1 mL of sample in tube to use for cell count. For counting with a Guava PCA, dilute 1:20 in insect cell medium (total of 400 μL), if the cell density is greater than  $0.5 \times 10^6$  cells/mL. If less than  $0.5 \times 10^6$  cells/mL, then dilute 1:10.

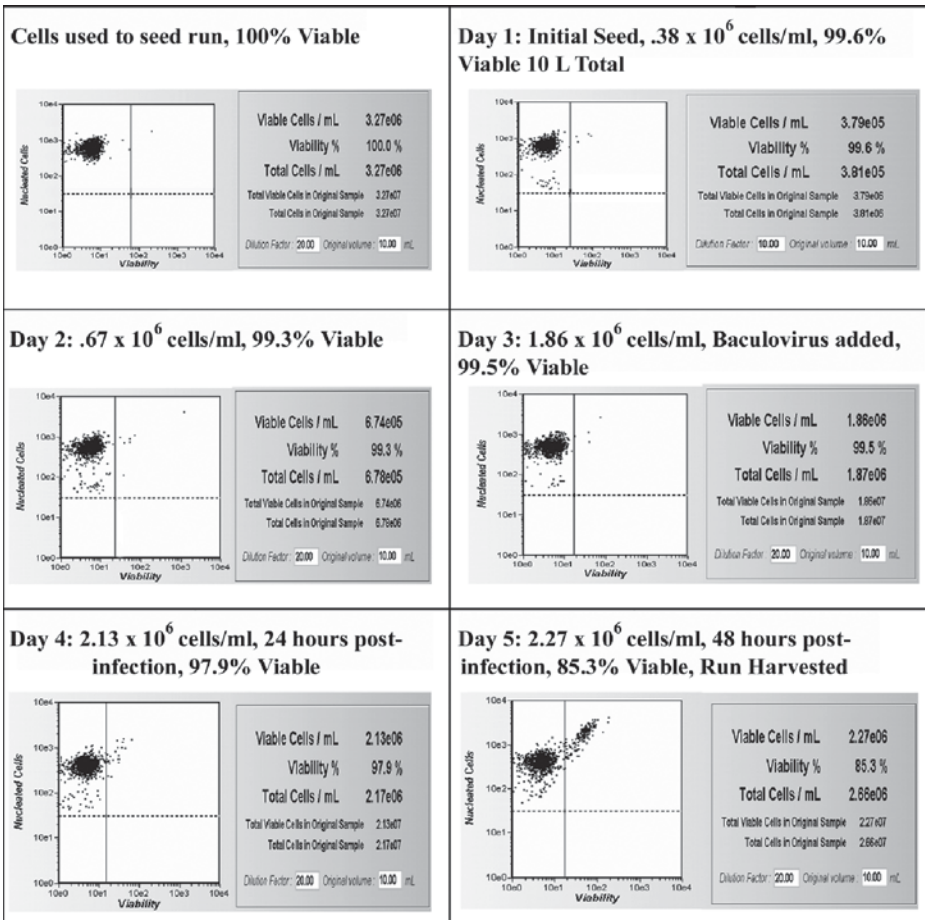


Fig. 4. Overview of Guava PCA data from a typical Cellbag20 Sf-9 production run. Viaflex reagent is used with this method of cell counting instead of trypan blue. The instrument differentiates viable from nonviable cells based on differences in permeability to the DNA-binding dyes in the Viaflex reagent.

Add 2  $\mu$ L Viaflex reagent, mix gently by vortexing, and count on Guava unit (*see Note 5*). An example of Guava cell analysis during a baculovirus production run in a wave cellbag is given in Fig. 4. For cell counts using the ViCell XR, a minimum of 0.5 mL culture is required in the sample cups provided. Select the appropriate cell type for the culture and start the analysis. Typically, 50 images are recorded of each sample, although this is configurable with fewer or more as required. Images or Excel data can be exported, again as required. A simple text file is generated for every sample by default. An example of Vi-Cell data gener-

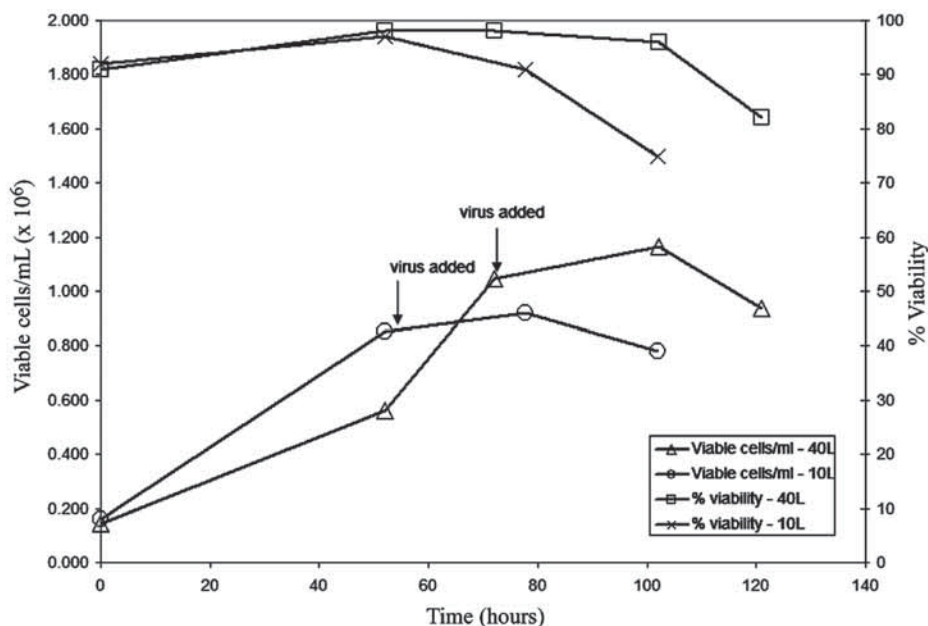


Fig. 5. ViCell data showing viable cell counts per milliliter and percent viability for a typical baculovirus infected culture (using a BioWave20 and a BioWave200, using 10 and 40 L working volumes, respectively).

ated during production is given in [Fig. 5](#). A feature of the Vi-Cell is the measurement of cell size. Insect cells increase in size after virus infection, and this can be tracked as an indicator of infection ([Fig. 6](#)).

### 3.6. Harvesting

1. Cells are harvested using a CARR ViaFuge Pilot 9004, following the manufacturer's installation specifications and procedures. To begin the harvest, rocking is stopped on the Wave unit. If using DO probes, then remove from the cellbag and store properly. Disconnect the airflow line and unclamp all filters.
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 harvest rigging set was made to our specifications by SAFC to quick-connect to the harvest line on the Cellbag100s and 200s ([Fig. 7](#)). For the Cellbag50s, using the Sterile Tube Fuser 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 this same rigging set.
4. The rigging set provides a length (7 ft) of silicon tubing (1/2" ID × 3/4" OD) for use with a peristaltic pump, to move the cell culture from the cellbag into the

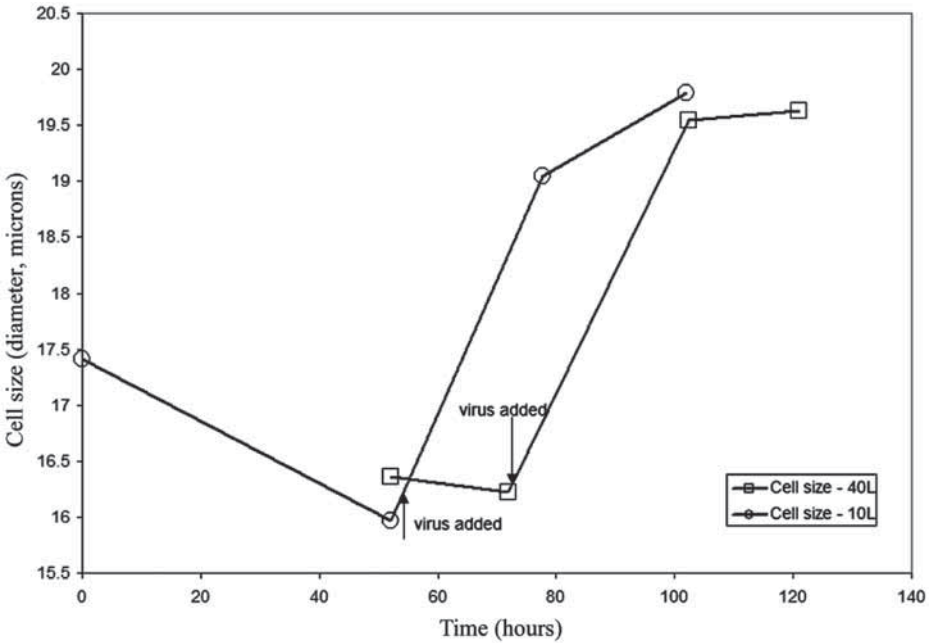


Fig. 6. ViCell data showing average cell size diameter, showing the typical cell swelling postviral infection (using a BioWave20 and a BioWave200, using 10 and 40 L working volumes, respectively).

prechilled Viafuge. This is connected to the Viafuge feed hose via a 1/2" Colder barb-lock mini-sanitary fitting and a triclover clamp.

5. The rigging set also provides two 3' lengths of 3/8" ID  $\times$  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 1X 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, then the medium is collected from the Viafuge concentrate outlet.
7. If the production run is for an intracellular protein, then the Viafuge concentrate outlet is connected via tubing to a decontamination tank.
8. The Viafuge process setting used is 5000 rpm (approx 2000g) with an input pump flow rate of 1.8 L/min. *It is important to determine the process setting and the pump flow rate specific to the type pump and tubing used, as well as the cell type and density.*
9. To remove cell concentrate from the Viafuge bowl, the pump speed is stopped and reversed to transfer into a harvest bottle held on ice. The volume of the

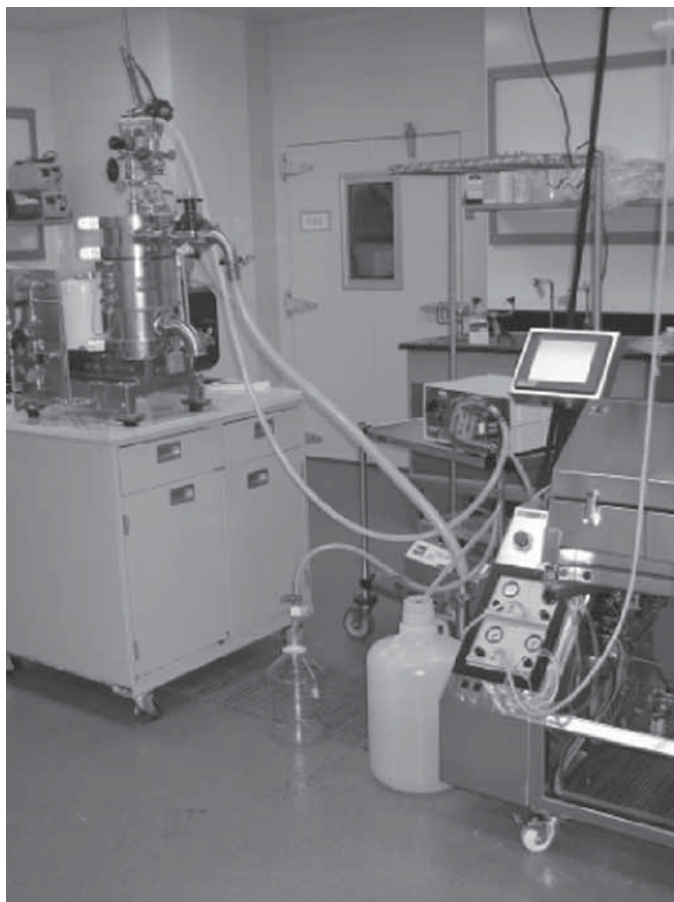


Fig. 7. Harvest rigging set shown interfacing the Wave System200 (tilted into harvest position), the peristaltic pump, the Viafuge, buffer wash carboy, and harvest bottle.

Viafuge bowl is 1.8 L. Care is used to keep harvested cells chilled to minimize protein degradation as a result of protease activity.

10. When the cell concentrate has been pumped into the bottle, the Viafuge bowl is washed with 1X PBS and the contents of the bowl are again collected.
11. Cells from the collection bottles are poured into Nalgene 1-L centrifuge bottles and centrifuged at 900g and 4°C for 30 min. The supernatant is poured from the cells and the pellets combined into one bottle and washed with chilled PBS. Any desired protease inhibitors can be added to the PBS. The pellet is centrifuged at 900g as previously described, 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 as it is filled. It is snap frozen (e.g., in an ethanol-dry ice bath or in liquid N<sub>2</sub>). Pellets are weighed and stored at -80°C (see **Note 7**).

12. If the desired protein is secreted, then it can be harvested with the Viafuge and filtered to remove cell debris. If necessary, the supernatant can be concentrated before purification. Filters used during filtration and concentration should be tailored to the protein expressed.

### 3.7. Product Quality Control

The standard methods for analyzing protein production quality and quantity include Coomassie stained sodium dodecyl sulfate-polyacrylamide gels and Western blots. As in many laboratories today, most of the recombinant proteins we express include tags for purification purposes. We make use of these tags for small-scale analysis when possible, 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.

If the recombinant protein is secreted, then a 5-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, which should contain the secreted recombinant protein, can be used for loading onto gels or for small-scale purification as previously described. The cell pellet can also be lysed to check for intracellular recombinant protein.

If the conditioned medium is to be concentrated, then samples should be collected during this process to ensure the desired recombinant is present in the concentrate and not lost in the permeate (these collected samples should be analyzed before the permeate is discarded).

The purified protein can also be analyzed by mass spectrometry and protein sequencing. Liquid chromatography–mass spectrometry/mass spectrometry is available to our group, and this provides excellent confirmation data.

In many cases, an activity assay can be used for confirmation of expression. Small-scale samples can be tested from purification preparations or possibly even tested at the lysed cells or conditioned medium stage without purification.

## 4. Notes

1. If the System20 or 50 units are used outside of a temperature controlled room or cabinet, then attention to condensation buildup on the filters is important. Monitor the air flow and watch for high pressure indicating filter plugging by conden-

- sation. Use a Kimwipe or tissue to remove any condensation on the external side of the filters. Lids for the units are also available for purchase.
2. If it is difficult to feel airflow through a restrictor on a cellbag, then it should be tested during set up. Remove the restrictor and attach to a syringe via the luer lock. You should be able to force air through the restrictor. Occasionally, these are totally plugged, and will need to be replaced.
  3. A fiber optic dissolved oxygen monitor and probe is also available from Wave Biotech. With this system, a fiber optic oxygen microsensor is inserted into a modified Oxywell port on the cellbag.
  4. Instead of purchasing custom made bags for liquid addition to the cellbags, addition vessels ("aspirator bottles") used can be obtained from Corning. A stopper with a middle bore is placed on the top port with a filter (Acro 50 Vent Filter P/N 4251, which can be obtained from Pall Filters) inserted into the stopper bore. The filter and stopper are wrapped with a milk filter, then with aluminum foil. Autoclave tape is used to secure the filter top. Approximately 2 ft of C-flex tubing is attached to the bottom dispenser port and the open end is wrapped with an isotropic milk filter (Schwartz Manufacturing Co., Two Rivers, WS) plus foil and secured with autoclave tape. Addition vessels for virus transfer into cellbags are set up with 1/8" ID  $\times$  1/4" OD C-flex on the bottom dispenser port to match an available line on the cellbag. For cell addition, 1/4" ID  $\times$  7/16" OD C-flex tubing is used. The prepared addition vessels are autoclaved using a "PreVac" cycle for 1 h with a 15-min dry time.
  5. As the units purchased from Wave Biotech (United States) and Wave Biotech AG (Switzerland) differ in their dimensions and design, values are listed for the US units and the Swiss units, with the Swiss units in parenthesis. The US System200EH has a maximum rock angle of nine, whereas the maximum rock angle for the Swiss unit is 12.
  6. In our hands use of the Guava ViaCount Reagent, normally used to count mammalian cells with the Guava PCA, resulted in low viabilities. The Viaflex reagent is recommended for insect lines. Also, cells are diluted in insect cell medium and not PBS for counting.
  7. An alternative to centrifugation into 1-L Nalgene bottles is to use Beckman Coulter HarvestLine System Liners (part number 369256, or kit 369264, *see* <http://www.beckman.com/>), which fit inside the adaptors for the H-6000A rotor (part number 00511, *see* <http://www.kendro.com/>). Used according to the manufacturer's instructions, it is possible to harvest cells directly into a preweighed flat bag that facilitates freezing and thawing. Manual pipetting or aspiration can be used to remove the wash buffer. The bags can then be reweighed to give the total pellet weight, and frozen as described in **Subheading 3.6., step 11**. The one-way vent in the neck of the bag prevents cells from leaking out.
  8. Other disposable cell culture systems are available, although not used in GSK laboratories to date. For example, *see* <http://www.metabios.com/>, <http://www.applikon.com/>, or <http://www.sartorius.com/index.php?id.3977>.

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## Protein Production With Recombinant Baculoviruses in Lepidopteran Larvae

Yi Liu, Nathan DeCarolis, and Nikolai van Beek

### Summary

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 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.,  $\alpha$ -interferon [1], mouse interleukin-3 [2]), and this host insect continues to be extensively used both for research (e.g.,

murine IgG [3], porcine interleukin-18 [4], ovine interferon  $\tau$  [5] and for commercial protein production, e.g., by the Japanese firm Katakura (e.g., human chymase (6), and bovine interferon  $\tau$  [7]). Other lepidopteran hosts have also been investigated, including the tobacco hornworm *Manduca sexta* (8), the cecropia moth *Hyalophora cecropia* (9), the beet armyworm *Spodoptera exigua* (10), the tobacco budworm *Heliothis virescens* (11), the saltmarsh caterpillar *Estigmene acrea* (12), and, probably most importantly, the cabbage looper *Trichoplusia ni*.

*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is by far the most widely used baculovirus expression vector. Although AcMNPV infects a wide range of lepidopteran hosts, not all moths are good hosts for this virus. For example, although being susceptible to a mutant (13,14), *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* (15). *T. ni* is an excellent host for AcMNPV and has been extensively used to produce a variety of proteins (e.g., human receptor kinase domain [16], human adenosine deaminase [17], human Group II Phospholipase A2 [18], interleukin-2 [19,20], and the cardiac sodium-calcium exchanger [21]).

Although commercial-scale protein production in *T. ni* larvae is available (Chesapeake PERL, Savage, MD [www.c-perl.com], and Entopath, Easton, PA [www.entopath.com]), this chapter is aimed at small to medium scale production on the bench-top. Compared to production in cultured insect cells, larvae can produce protein at reduced cost and without the need for investment in expensive equipment. In addition, the scale-up issues typical of reactor-based production do not occur when using insect larvae.

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 (*T. ni* or *S. frugiperda* cells), followed by amplification of budded virus particles, and establishment of an infection by injection of 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 preoccluded 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 *T. ni* Larvae

1. We recommend the purchase of eggs or larvae from any of several commercial suppliers (Benzon Research, Carlisle, PA, [www.benzonresearch.com](http://www.benzonresearch.com); Bio-Serv, Frenchtown, NJ, [www.bio-serv.com](http://www.bio-serv.com); Entopath) (*see Note 1*).
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 (Bio-Serv, cat. no F9772), a dry mix insect diet (*see Note 2*). We recommend purchase of insect eggs and diet from the same vendor.
4. Transfer forceps (e.g., BioQuip, Rancho Dominguez, CA; cat. no. 4750): soft tweezers for handling of insect larvae.
5. 8-oz Cups and fitting lids (e.g., Solo Cup Company, Urbana, IL) for rearing of larvae in groups of approx 25 individuals (*see Note 3*).
6. Dissecting microscope with a micrometer scale engraved in one ocular lens (*see Note 4*).

### 2.2. Inoculation by Injection

1. *Spodoptera frugiperda* Sf-9 (ATCC, cat. no. CRL-1711) or Sf-21 cells can be used for generation of recombinant virus, as well as for titration of the extracellular virus stock (*see Note 5*).
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 Note 6*) (*see Chapter 8*).
3. 10- $\mu$ L Syringe (Hamilton, Reno, NV), fitted with a 26s-gauge needle.

### 2.3. Oral Inoculation

1. Mortar and pestle.
2. FD&C Blue no. 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 (Invitrogen): a transfection reagent.
2. Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI).

### 2.5. Homogenization and Clarification

1. Antimelanizing agent: either  $\beta$ -mercaptoethanol (BME) (Sigma-Aldrich, St. Louis, MO), or a stock solution of 25 mM phenylthiourea (PTU) in ethanol (Sigma-Aldrich) (*see Note 7*).
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 (~approx 29°C), either in the dark or under a day/night regimen, they develop from egg to pupa in approx 13 d. After about 2 d in the egg stage, larvae will hatch and progress through five instars over a period of about 9 d. Late in the fifth instar, larvae change in appearance when they enter the prepupal stage, which lasts approx 2 d. Metamorphosis takes place in the pupal stage, and adult insects emerge after about 4 d.

##### 3.1.1. 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 approx 37°C, pour approx 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 wk without any detrimental effects. Remember to equilibrate the diet to room temperature for at least 1 h and let condensation water evaporate before placing insects or eggs on the diet.

##### 3.1.2. *T. ni* Rearing

1. *T. ni* can be purchased as surface-sterilized eggs attached to a substrate such as paper towel or muslin (**Subheading 3.1.2.1.**), as "loose" eggs (**Subheading 3.1.2.2.**), or as larvae on diet (**Subheading 3.1.3.**).

###### 3.1.2.1. LARVAL REARING STARTING WITH EGGS ON A SUBSTRATE

1. Cut the substrate into small strips (containing between 50 and 100 eggs). Egg density can be estimated by counting under a dissecting microscope. To this end mark an area of approx 1 in.<sup>2</sup> 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 d of incubation, begin to monitor larval development as described in **Subheading 3.1.3.**

#### 3.1.2.2. 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/corn 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 approx 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 d of incubation, begin to monitor larval development as described in **Subheading 3.1.3.**

#### 3.1.3. Determination of Developmental Stage

*T. ni* larval development consists of five instars, which are developmental stages separated by a molt. Because injection of larvae is carried out preferentially at 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 d 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**).
2. Estimate the number of larvae per cup and reduce to approx 25 (*see Note 8*).
3. At 29°C it will take 6–7 d between seeding of the eggs and the molt from fourth to fifth instar (*see Note 9*). 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.

### 3.2. Recombinant Virus

The construction of recombinant baculovirus vectors is described elsewhere in this book (Chapter 4), and a number of different systems are available from

**Table 1**  
**Weight and Head Capsule Width of *Trichoplusia ni* Larvae**

Insect stage (instar)	1st	2nd	3rd	4th	5th
Head capsule width ( $\mu\text{m}$ )	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

various commercial sources (e.g., Invitrogen, BD Biosciences/Pharmingen, Novagen, NextGen Sciences, and AB Vector). The kits vary in the manner in which the heterologous coding sequence is transferred into the baculovirus (e.g., homologous recombination, transpositional insertion), the ease of the procedures (screening), flexibility (number and types of expressible promoters, availability of purification tags, and secretion signals, and so on), or even in the presence/absence in the vector of a virally encoded protease. However, the choice of a particular system is not influenced by whether the recombinant virus will be used in cultured cells or in larvae; each system will yield recombinant AcMNPV suitable for infection of *T. ni* larvae and the expression of recombinant protein (see **Note 10** for safety aspects of recombinant baculoviruses).

### 3.3. Inoculation

There are three different methods for inoculating larvae: (1) injection with extracellular virus, (2) oral inoculation, and (3) 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 POV inoculum consists of virions, which are produced late in the infection cycle and which remain in the nucleus as they are destined to be incorporated into polyhedral occlusion bodies (22–24). 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 23).

2. **POV:** 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 11*). DNA is purified from the bacterial culture using a miniprep method, and DNA concentration in the inoculum can be estimated, e.g., by spectroscopy.

### 3.3.1. 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 approx 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 d, but in virus-infected larvae pupation (in fact, any molt) is blocked by a virus-encoded ecdysteroid UDP-glucosyltransferase (25). Infected larvae will die from the virus infection after approx 3–3.5 d (or, if lower doses are used, after as long as 4–5 d).

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 approx 10 min before inoculation, when they may be cooled on ice in groups of five larvae (*see Note 12*).
3. Inject four groups of five larvae with 1  $\mu$ L 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  $\mu$ L 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 approx 24 h, remove any larvae that died from the injection procedure.

7. Harvest infected larvae when approx 10% of the remaining insects are dead, typically between 72 and 84 h after inoculation. If the larvae are not to be processed immediately, they should be stored at  $-80^{\circ}\text{C}$ .

### 3.3.2. Oral Inoculation

1. Follow the procedure for injection of larvae as outlined in **Subheading 3.3.1.**, but inject only 20 larvae with viral inoculum.
2. Harvest the infected larvae approx 3.5 d after inoculation.
3. Freeze the larvae at  $-20^{\circ}\text{C}$ .
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 approx 700 mL of a solution of blue dye in water, using either 0.5 mg/mL FD&C no. 1 or approx 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 approx 4–5 d, when approx 10% of them have died from the virus infection. If the larvae are not to be processed immediately, they should be stored at  $-80^{\circ}\text{C}$ .

### 3.3.3. 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: (1) it eliminates the need to set up and maintain a system of cultured insect cells, and (2) it cuts almost 1 wk off the time between cloning of the heterologous gene and expression of recombinant protein in *T. ni* larvae (see **Note 13**).

Because 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 (**26**).

1. Amplify by PCR a fragment containing the open reading frame of DsRed from pDsRed (BD Biosciences/Clontech, Palo Alto, CA), adding a *Bgl*II and a *Bsp*I restriction enzyme site 5' and 3' of the open reading frame, respectively.
2. Digest the resulting fragment of approx 700 bp with *Bsp*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  $\mu$ L Cellfectin and 15  $\mu$ L 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  $\mu$ L Cellfectin, 10  $\mu$ L miniprep DNA (1 mg/mL), and 50  $\mu$ L Sf-900 II medium and let sit at room temperature for at least 15 min before use.
8. Select approx 100 early fifth instar *T. ni* larvae as in **Subheading 3.1.3**.
9. Inject 20 larvae with 1  $\mu$ L control inoculum each, and 80 larvae with 1  $\mu$ L transfection inoculum each, as described in **Subheading 3.3.1**.
10. Incubate the larvae, and remove any dead larvae after 24 h as described in **Subheading 3.3.1**.
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%, whereas mortality caused by the injection procedure was 10% (see **Note 13**).
12. Store harvested larvae at  $-80^{\circ}\text{C}$  until processing.

### 3.4. 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 toward the end of the infection cycle. The ideal time to harvest is different for each larva, because 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 approx 10% of the population has died.

### 3.5. 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 nonsoluble 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  $\mu$ M PTU (1/1000 vol 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. 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 protein free of mammalian components it may be necessary to ascertain that no animal-derived components are included in the diet.
3. 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 2 and 3 per in<sup>2</sup> diet surface.
4. A balance can also be used to determine the larval development stage. However, measurement of head capsule width under a dissecting microscope is the more reliable method.
5. 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 either of the two insect cell types and for virus titer determination (TCID<sub>50</sub> or overlay plaque assay) can be found elsewhere in this book (Chapter 9 and Chapters 4, 5, 10, 11, and 21, respectively).
6. Serum-containing and serum-free media are available for these cell lines. Because 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.

7. 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 BME and 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.
8. 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.
9. 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.
10. 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 National Institute of Health classifies experiments with recombinant baculoviruses in the lowest Risk Group, RG1. Material contaminated with recombinant baculovirus should be autoclaved before disposal.
11. 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.
12. Injection can be done without cooling the larvae; in fact, if one has acquired some experience, it is faster.
13. A similar method has recently been described for the transfection of *B. mori* larvae (27).
14. By injecting fourth-instar instead of fifth-instar larvae, we achieved infection in more than 90% of the larvae. However, because of their smaller size, injecting these larvae was more tedious and led to significantly higher control mortality.

## Acknowledgment

We thank Ian Smith (Nara Institute of Science and Technology, Japan) for reviewing the manuscript.

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## Virus-like Particle and Viral Vector Production Using the Baculovirus Expression Vector System/ Insect Cell System

*Adeno-Associated Virus-Based Products*

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### Summary

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 candidate vaccines, are currently being addressed. Furthermore, these VLPs are being investigated as delivery agents for use as therapeutics. Recently, adeno-associated viral (AAV) vectors, which can be potentially used for human gene therapy, have been produced in insect cells using three baculovirus vectors to supply the required genes. The use of host insect cells allows mass production of VLPs in a proven scalable system. This chapter focuses on the methodology, based on the work done in our lab, for the production of AAV-like particles and vectors in a BEVS/insect cell system.

**Key Words:** Viral vector; viral-like particle; virus-like particle; adeno-associated virus; 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 that 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

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

can perform most of the post-translational modifications of mammalian cells (see Chapter 17), 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. This includes work on blue-tongue virus (1,2), rotavirus (3–6), human (2,7) and porcine (8–11) parvoviruses, human immunodeficiency virus (12,13), and infectious bursal disease virus (14–16).

Viral vectors produced in insect cells are a natural extension to the production of VLPs. 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. Recently, following the initial reports that adeno-associated viral (AAV) vectors could be produced in insect cells (17), Meghrou et al. (18) investigated the scale-up of the production of these vectors up to the 20-L scale. AAV VLPs produced in insect cells have been previously studied using multiple baculoviruses coding for the individual structural proteins (19,20); however, the consistency of the composition of the particles were found to be highly dependant on the infection conditions, e.g., the multiplicity of infection (MOI). Urabe (17) developed a single baculovirus vector that allowed the expression of all three structural proteins (viral protein [VP]-1, VP2, and VP3).

This chapter describes a methodology for producing and monitoring adeno-associated VLPs and viral vectors using the BEVS with a host insect cell. The AAV is used in this chapter as an example and the methodology is equally applicable to any VLP or viral vector type. Also, any production strategy previously described in this book and particularly in Chapters 10–12 is similarly applicable to the production of AAVs and VLPs.

## 2. Materials

### 2.1. Cell Lines and Recombinant Viruses

#### 2.1.1. Production

1. *Spodoptera frugiperda* cell line Sf-9 (ATCC CRL1711) (see **Note 1**).
2. Recombinant *Autographica californica* multiple nucleopolyhedrovirus (AcMNPV) (see **Note 2**): (1) with the Cap gene (BacCap), (2) with the Rep genes (BacRep), and (3) with the gene of interest flanked by AAV ITRs (BacITRTransgene).

#### 2.1.2. Infectious Assay of the AAV

1. HEK 293 cell line (Invitrogen, Carlsbad, CA).
2. Human adenovirus type 5 (ATCC VR-1516).

## 2.2. Medium and Solutions

1. Insect cell culture medium (for Sf-9 cells): 9.5 kg H<sub>2</sub>O, 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<sub>3</sub>) solid, then add H<sub>2</sub>O to 10 L. Final pH should be 6.2 ± 0.1. Adjust pH if necessary with HCl (12 *N*). Final osmolarity should be 350 ± 25 mOsm. Other serum-free media could be used with generally similar growth performances (*see* **Note 3** and Chapter 8).
2. Mammalian cell culture medium (for HEK 293 cells): low calcium serum-free medium (LC-SFM) derived from HSFM (Invitrogen) supplemented with 0.1% Pluronic® F-68 (Invitrogen), 1% bovine calf serum (Hyclone, Logan, UT), 10 mM HEPES (Sigma, St. Louis, MO), 50 µg/mL G418 (Invitrogen). Final pH should be 6.95 ± 0.05. Adjust pH if necessary with HCl (12 *N*).
3. Trypan blue solution (0.04%).
4. Erythrosin B solution diluted to 0.025% (Sigma).

## 2.3. Specialty Kits/Products for AAV or Gene Detection

1. DetectaGene Green CMFDG *lacZ* Gene Expression Kit (Molecular Probes/Invitrogen, cat. no. D-2920).
2. Anti-AAV VP1, VP2, and VP3 antibodies (Maine Biotechnology Services, Portland, ME; cat. no. MAB659P).
3. ELISA kit (ARP American Research Products, Inc., Belmont, MA; cat. no. PRATV).
4. Anti-AAV Rep78, 68, 52, and 40 antibodies (Maine Biotechnology Services, cat. no. MAB689P).

## 2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gels

1. β-Mercaptoethanol.
2. 5X Concentrated Tris-glycine running buffer: 800 mL of Milli-Q H<sub>2</sub>O, 15 g Tris, 72 g glycine, 5 g sodium dodecyl sulfate (SDS); complete to 1 L with H<sub>2</sub>O. 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, Mississauga, ON, Canada).
5. 4–15% Tris-HCl Ready Gels (Bio-Rad).

## 2.5. Western Blotting

1. Gel blot paper.
2. Nitrocellulose membrane.
3. PBS (10X): 800 mL H<sub>2</sub>O, 2 g KCl, 2 g KH<sub>2</sub>PO<sub>4</sub>, 80 g NaCl, 21.6 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O; complete to 1 L with H<sub>2</sub>O. Filter through a 0.45-µm membrane filter.

4. PBS-T (0.1%): 100 mL PBS (10X), 900 mL H<sub>2</sub>O, 1 mL Tween-20.
5. 5% Dried skim milk in PBS-T (0.1%): 5 g blotting-grade blocker non-fat dry milk, 100 mL PBS-T (0.1%); gently heat mixture while stirring until dissolved.
6. Towbin transfer buffer: 700 mL H<sub>2</sub>O, 3.03 g Tris, 14.41 g glycine, 200 mL methanol; complete to 1 L with H<sub>2</sub>O.
7. TRANS-BLOT® Semi-Dry Transfer Cell (Bio-Rad).
8. BM Chemiluminescence Blotting Substrate (POD, cat. no. 150694) (Roche Diagnostic Corp. Laval, QC, Canada).

## 2.6. Postharvest Solutions

1. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 2 mM MgCl<sub>2</sub>, 50 U Benzonase™/10<sup>7</sup> cells/mL lysis buffer.
2. 375 mM MgSO<sub>4</sub> stock solution.

## 2.7. Shake Flask and Bioreactor Culture

Shake flask cultivation (up to 600 mL working volume) is done in a 27°C temperature controlled incubator equipped with a shaker table. The agitation is normally set at 110 rpm for the production of AAV VLPs or AAV vectors.

For larger working volumes it is preferable to use a bioreactor as described in detail in Chapter 11. For example, at mid-scale, a 3.5- or 22-L Chemap bioreactor (Mannedorf, Switzerland) can be used. Because of 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 should 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 cell growth (*see* Chapter 11). The air, oxygen, and nitrogen flowrates into the bioreactor are controlled by mass flowmeters. Depending on the cell density of the culture, air, or oxygen sparging may be necessary; however, surface aeration is sufficient for cell densities up to  $2 \times 10^6$  cells/mL, which is an optimal cell density for AAV production.

## 2.8. Online Data Acquisition

Online data acquisition is considered optional, but to understand the dynamics of the process and to check reproducibility of the run, it is an asset. A computer with a data acquisition hardware/software that can record several signals simultaneously online should be installed, i.e., for temperature, DO, pH, RPM, gas flow rates, pressure, capacitance, and so on (*see* Chapter 11).

## **2.9. Offline Data Acquisition**

### *2.9.1. Cell Monitoring*

1. Hemacytometer (Hausser Scientific, Horshaw, PA).
2. Coulter Counter Multisizer II (Beckman Coulter, Inc.) (optional).

### *2.9.2. AAV Detection*

1. Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc.) (optional).
2. Spectrophotometer with a 450-nm filter
3. Kodak Image Station 440CF (Mandel Scientific Co., Ltd.)

### *2.9.3. Other Equipment*

1. Hot water bath or heating block.
2. A 37°C incubator equipped with a shaker table (110 rpm) and that can maintain a 5% CO<sub>2</sub> environment (for mammalian cells used for the AAV infectious assay).

## **3. Methods**

### **3.1. Insect Cells**

Before proceeding to the preparation of viral stocks and AAV VLP or vector production, make sure that the cells are in good shape. This can be assessed by monitoring the doubling time of the cells, which should be around 24 h (specific growth rate around 0.03/h) (*see* Chapter 1). If a Coulter Counter Multisizer (or equivalent equipment) is available it is desired to make sure that the cell size distribution is as symmetric as possible and that the cell diameter be consistent with the cell diameter of non-infected cells. We have seen a reduction in AAV titer at cell passages greater than 40; therefore, it is recommended to use lower cell passages when producing AAV. Cell inocula can be done in shake flasks limiting the working volume to 20% of the total volume. Cells should be kept in the exponential growth phase by inoculating the cell culture at  $3 \times 10^5$  cells/mL and not letting the cells go beyond  $5\text{--}6 \times 10^6$  by transferring the cells in fresh media one to two times per week.

#### *3.1.1. Cell Count*

Viable and total insect cells are counted using a hemacytometer (Hausser Scientific) or by using a Coulter Counter Multisizer II. Insect cell viability is assessed by dye exclusion method using Trypan blue (*see* Chapter 11).

### **3.2. Baculovirus Stock Amplification**

The baculoviruses stocks are amplified using generally Sf-9 insect cells grown in suspension and serum-free media (many researchers prefer to add fetal bovine serum to increase virus stability, e.g., *see* Chapter 1). Preparation

of large baculovirus stocks is described in Chapter 11 in Subheading 3.2. In brief, Sf-9 cells in exponential growth (see Chapter 1) are infected at an MOI of approx 0.1 in shaker flasks. Infected cultures are further incubated and monitored daily. A decline in viability is observed after approx 48 h postinfection (pi). The cells are harvested when viability is around 80% and this is typically between 72 and 120 h pi. The cell suspension is centrifuged to remove cells and cell debris and the supernatant is sterile-filtered and stored at 4°C until further use (see Note 4). The baculovirus stock should be titered prior to use (see Chapters 4, 5, 10, 11, and 21).

### **3.3. AAV 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 next size bioreactor at  $0.3\text{--}0.5 \times 10^6$  cells/mL. It is recommended that the data acquisition system be set 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.

1. Add medium, preheated to 27°C, to the bioreactor.
2. Start the agitation at 80 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 40% air saturation.
4. Once the temperature and the dissolved oxygen concentration have stabilized cells can be added to the bioreactor such that the initial cell density is in the range of  $0.3$  to  $0.5 \times 10^6$  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. Culture Control and Monitoring*

During the cultivation, either for VLP or viral vector production, parameters such as dissolved oxygen concentration, agitator rotational speed, and temperature should be monitored and controlled. An example of the dynamics of these parameters being controlled is given in **Fig. 1**. Dissolved oxygen should be controlled at 40% air saturation. In shake flasks, loosening the lid and maintaining a low working volume to flask volume ratio (which provides a large liquid–gas interface) results in good oxygen transfer. Temperature should be maintained at 27°C. Because of the shear sensitivity of the cells, the impeller speed should be kept constant for the entire batch (*see Note 5*). Other parameters that are not controlled but that are generally monitored include signals that can

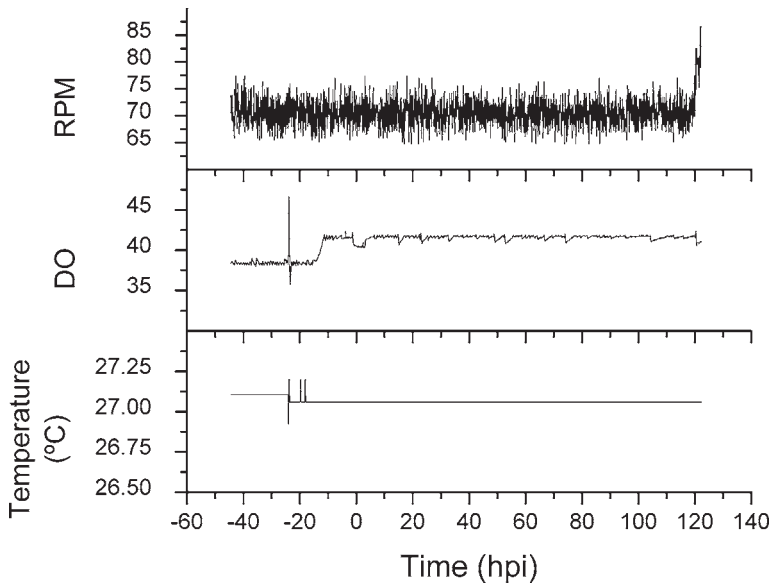


Fig. 1. Online data of controlled parameters in a 3-L bioreactor for the production of adeno-associated viral in the *Spodoptera frugiperda* Sf-9 cell line infected at  $3 \times 10^6$  cells/mL with equal amounts of BacCap, BacRep, and BacITRGFP at an overall multiplicity of infection of five.

be collected online or measured offline. Online parameters that are solely monitored may include signals from a fluorescence probe (e.g., if the green fluorescent protein [GFP] reporter gene is used, *see* Chapter 21) or a capacitance probe. Offline measurements may include cell density, cell viability, total VP concentration, infectious virus particle concentration, viral proteins (e.g., Rep52), and nutrient concentrations (e.g., glucose and glutamine). The dynamics of some of these parameters are shown in **Fig. 2**. Similar data can be collected for AAV VLP production. Typical dynamics of VLP production of cells infected at an MOI of five is shown in **Fig. 3**.

### 3.3.3. Production Modes

Grow the cells to  $1.5\text{--}2 \times 10^6$  cells/mL prior to infecting to provide cells in the exponential growth phase (*see* Chapter 1). Infection might be completed at higher cell densities if cells are infected in fresh medium under nonlimiting nutrient conditions. Yields can be increased by changing the medium prior to infection. Another approach is to operate under a fed-batch mode. For more details *see* Chapter 11.

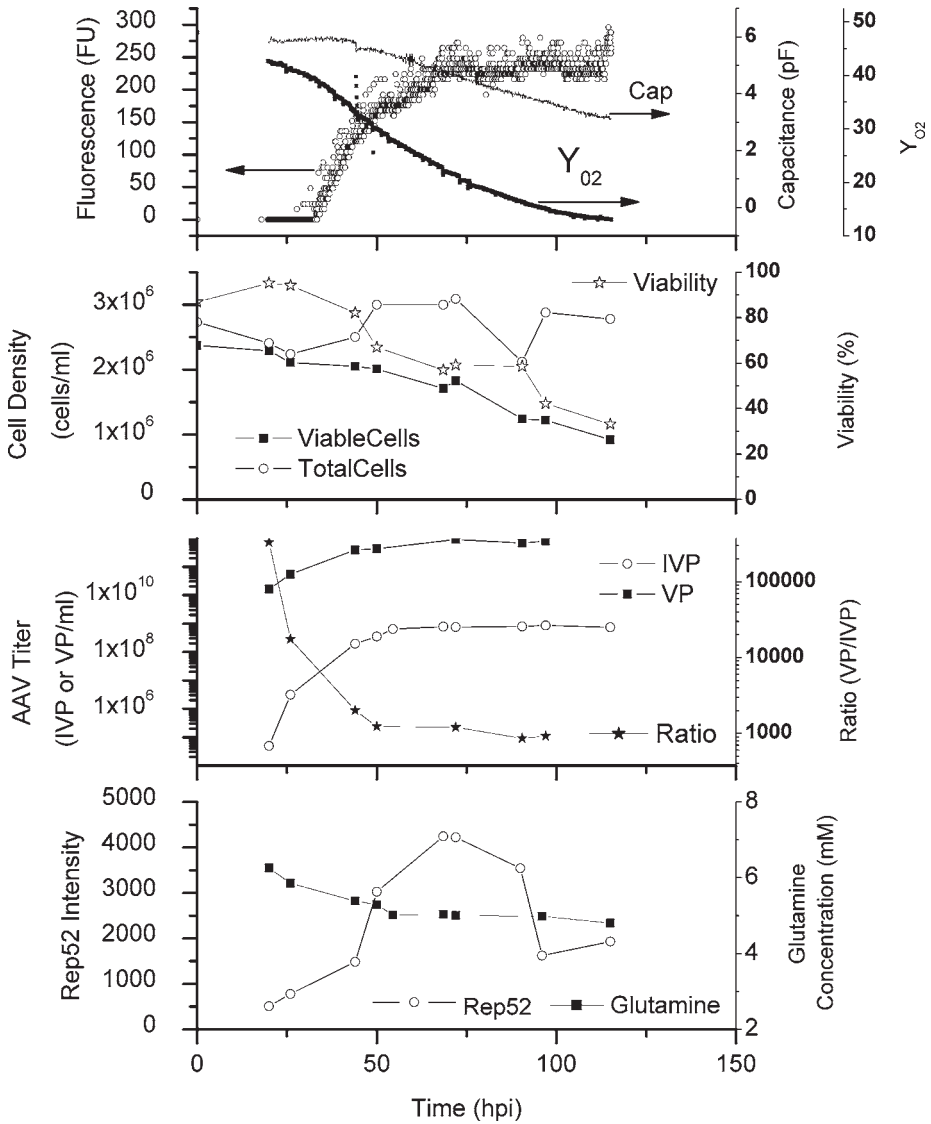


Fig. 2. Online and offline data of monitored parameters in a 3-L bioreactor for the production of adeno-associated viral in the *Spodoptera frugiperda* Sf-9 cell line infected at  $3 \times 10^6$  cells/mL with equal amounts of BacCap, BacRep, and BacITRGFP at an overall multiplicity of infection of five (for details see ref. 18). Y<sub>02</sub> is the percentage of oxygen in the inlet gas (which contains only O<sub>2</sub> and N<sub>2</sub>) that is required to maintain the culture setpoint of 40% air saturation.

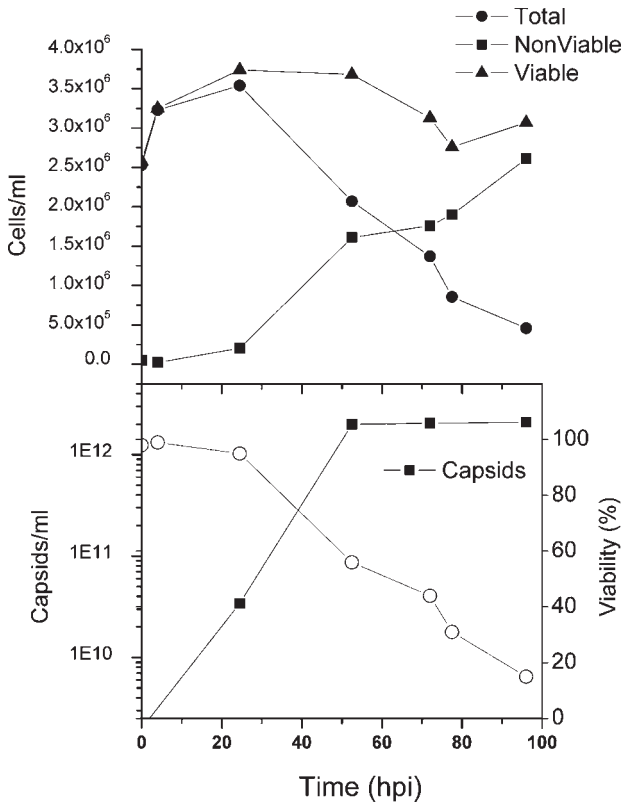


Fig. 3. Dynamics of adeno-associated virus-like particles production in the *Spodoptera frugiperda* Sf-9 cell line infected at  $2.5 \times 10^6$  cells/mL with BacCap at an MOI of five in Sf-900II serum-free medium.

### 3.3.4. Culture Infection

Infect the cells with an MOI of 5–10 for each baculovirus (BacRep:BacCap:BacITR of 1:1:1). High MOIs lead to a synchronous infection and faster AAV production 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 make sure not to infect a cell culture with more than 10% viral stock volume to total volume. In some cases, concentration of the virus stocks may be necessary. This can be done for small volumes (<100 mL) by ultracentrifugation at 48,000g for 2 h at 4°C and resuspending the pellet in medium (22) or by tangential flow filtration for larger volumes. For more details see Chapter 11.

### 3.3.5. Culture Harvest and AAV Extraction

The optimal time to recover AAV in the cell pellet is at 72 h pi before significant cell lysis occurs (see **Note 6**).

The procedure to extract AAV from the cell culture is as following:

1. Harvest the cells by centrifuging at 325g for 15 min at 4°C.
2. Discard the supernatant.
3. Resuspend the cell paste in lysis buffer.
4. Incubate for 30 min at room temperature with gentle agitation.
5. Add MgSO<sub>4</sub> stock solution to get a final concentration of 37.5 mM.
6. Incubate for 30 min at room temperature with gentle agitation.
7. Freeze the lysate in dry-ice/ethanol bath.
8. Thaw in water bath at 37°C.
9. Freeze second time in dry-ice/ethanol bath.
10. Thaw second time in water bath at 37°C.
11. Freeze third time in dry-ice/ethanol bath.
12. Thaw third time in water bath at 37°C.
13. Centrifuge at 625g for 15 min at 4°C.
14. Filter supernatant through 0.45- $\mu$ m filter.
15. Purify AAV either by chromatography or a combination of iodixanol (**23**) gradient centrifugation and chromatography methods.

### 3.3.6. Analytical Methods

#### 3.3.6.1. GENE TRANSFER ASSAY (SOMETIMES REFERRED TO AS TRANSDUCTION OR INFECTIOUS ASSAY)

Infectious AAV-2 production is determined by transducing HEK293 EBNA cells as follows:

1. Subject Sf-9 cell culture samples to three freeze/thaw cycles.
2. Heat cell lysates at 60°C for 15 min in a water bath or heating block to inactivate the baculovirus.
3. Transfer 1 mL of HEK 293 cells, infected with wild-type adenovirus at an MOI of 50, to each well of a 12-well culture plate to obtain  $5 \times 10^5$  cells/well.
4. Coinfect cells with serial dilutions of cell lysate (obtained in **step 2**) in LC-SFM medium.
5. Incubate at 37°C for 24 h on a circular shaker rotating at 110 rpm in a 5% CO<sub>2</sub> environment.
6. Harvest the cells by centrifugation (2 min, 500g and resuspend in 1 mL of PBS buffer and fix with 2% paraformaldehyde) (see **Note 7**).
7. Use cells infected with only adenovirus as the negative control.
8. Select the dilutions corresponding to transduction between 2 and 30% of the cells (that falls within a linear response range).
9. Number of cells infected corresponds to the number of viral vectors present in the diluted sample added in **step 4**. Calculate the number of viral vectors present in undiluted sample.

### 3.3.6.2. FACS ANALYSIS (FOR FLUORESCENT MARKERS)

To analyze infected Sf-9 and HEK 293 cells expressing GFP, a Coulter Epics Profile II equipped with a 15-mW air-cooled argon-ion 488 nm laser may be used (*see Note 8*). The flow cytometric data may be analyzed using the EXPO32™ software package.

1. For each sample, determine the percentage of fluorescent cells by flow cytometry by passing a minimum of 10,000 cells.
2. Detect the fluorescence signal by using appropriate filter combinations. For example, a 550-nm long pass dichromic filter and a 525-nm band pass filter is used to detect the fluorescence signal of GFP. Light scatter values are measured on a linear scale of 1024 channels and fluorescence intensities on a logarithmic scale.
3. Calibrate the flow cytometer using standard fluorospheres (Coulter, Miami, FL) corresponding to the size of the cells.

This method has also been adapted for the detection of  $\beta$ -galactosidase. The reagents are available commercially in the DetectaGene Green CMFDG *lacZ* Gene Expression Kit (Molecular Probes, cat. no. D-2920). After infection and incubation of the cells for approx 24 h, the cells are loaded with CMFDG substrate via the Influx Reagent/Hypotonic Shock method as described in the commercial kit. The CMFDG is cleaved by  $\beta$ -galactosidase producing a fluorescent product. The cells are then fixed using a 2% formaldehyde solution and detected by FACS under the same detection conditions as for GFP (*see Note 8*).

### 3.3.6.3. SDS-POLYACRYLAMIDE GELS/WESTERN ANALYSIS

To assess the composition of the particles, the individual viral proteins can be assessed using specific antibodies. The antibody used to detect the structural proteins is a monoclonal antibody against adeno-associated virus VP1, VP2, and VP3 (Maine Biotechnology Services, cat. no. MAB659P).

To monitor the replication proteins in culture (**Fig. 3**), Western blot analysis can be performed using antibodies against the replication proteins. In this system, Rep 52 is easily detected; however, Rep 78 is not over-expressed and is therefore harder to detect. Rep 40 and Rep 68 are not detectable either. The antibody used to detect the replication proteins is a monoclonal antibody against Rep 78, 68, 52, and 40 (cat. no. MAB689P, Maine Biotechnology Services). A typical SDS-polyacrylamide gels/Western blot of cell culture at 72 h pi is shown in **Fig. 4**.

1. Subject cell samples to three freeze–thaw cycles.
2. Add 36  $\mu$ L  $\beta$ -mercaptoethanol to 300  $\mu$ L of sample buffer.
3. Dilute culture samples 2:1 in sample buffer.
4. Heat samples in boiling water bath for 5 min.
5. Centrifuge samples at 16,000g for 2 min.
6. Load supernatant on gel.

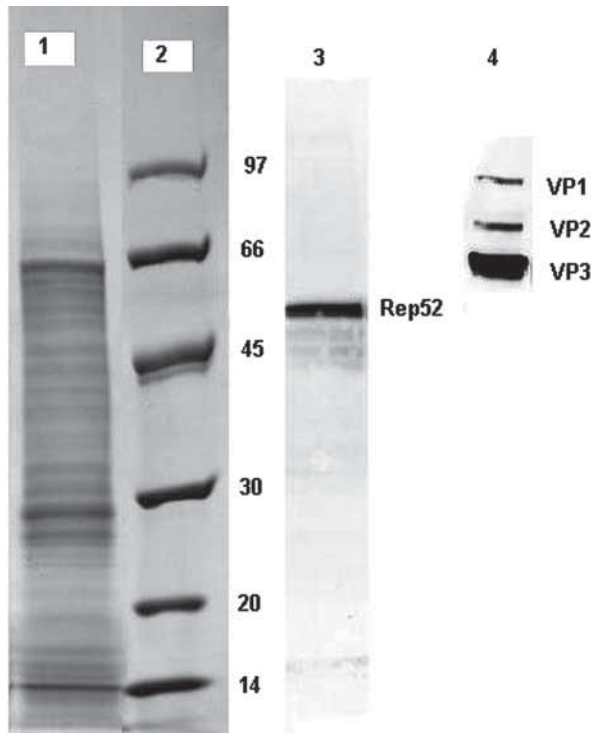


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gels (SDS PAGE)/Western blot of a sample from the cell culture at 72 h pi. The *Spodoptera frugiperda* Sf-9 cell line was infected with an MOI of five of each of the three baculoviruses. Lane 1 is a Coomassie Blue stain of the SDS-PAGE gel. Lane 2 contains the molecular weight markers. Lane 3 is the detection of the replication proteins and Lane 4 is the detection of the structural proteins by Western blot.

7. Run gel at 70 V for 15 min followed by 140 V for 60 min.
8. Stain with a Coomassie Blue solution or continue to **step 9** for Western blot analysis.
9. 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.
10. Prior to the end of the electrophoresis run, soak the blot paper and nitrocellulose membrane (NC), all in one container with Towbin Transfer buffer for 15–30 min (keep it at 4°C), in the following order: (1) 2 × blot paper, (2) 1 × NC, and (3) 2 × blot paper.
11. 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): (1) 2 × blot paper, (2) 1 × NC membrane, (3) gel (avoid moving the gel against the NC membrane once it is laid down), and (4) 2 × blot paper.

12. Carefully place the cathode assembly onto the stack.
13. Run at 10 V for 60 min.
14. Once the transfer is complete remove the NC membrane and let dry for 5 min.
15. Block in 5% dried skim milk/PBS-T (0.1%) for 1 h at room temperature with shaking.
16. Wash the membrane with PBS-T (0.1%): (1) 1 × 15 min and (2) 2 × 5 min.
17. Add either monoclonal anti-VP or anti-Rep diluted 1:1000 in PBS-T (0.1%).
18. Incubate overnight with shaking.
19. Wash the NC membrane with PBS-T (0.1%) with shaking: (1) 1 × 15 min and (2) 2 × 5 min.
20. Add the conjugated secondary antibody: anti-mouse IgG peroxidase conjugate in PBS-T (0.1%) and incubate for 1 h at room temperature with shaking.
21. Wash the NC membrane with PBS-T (0.1%) with shaking: (1) 1 × 15 min and (2) 4 × 5 min.
22. Detect using BM Chemiluminescence Blotting Substrate and analyse using a Kodak Image Station.

#### 3.3.6.4. ELISA

The total viral vector particles are assessed using cell lysate and a commercially available ELISA kit (ARP American Research Products, Inc., cat. no. PRATV). This kit is based on a sandwich ELISA technique and uses a mouse monoclonal antibody (A20) specific for a conformational epitope on assembled AAV-2 particles.

1. Freeze/thaw 3X cell culture sample.
2. Proceed with instructions from the ELISA kit.

## 4. Notes

1. AAV production can be done using Sf-9 or Tn-5 cells (18); however, use of Tn-5 cells results in needing to maintain two cell lines because the viral stocks need to be made in Sf-9 cells. It is our opinion that using only Sf-9 cells simplifies the production of AAV vectors.
2. Baculoviruses were obtained from Dr. Robert Kotin at the National Institutes of Health (Bethesda, MD). Other combinations can also be used for the production of rAAV, i.e., BacRep can be replaced by two baculoviruses: one coding for BacRep52 and the other for BacRep78. These were kindly donated by Dr. Zoluthukhin of the University of Florida.
3. For the production of AAV viral vectors, we have also used EX-CELL™ 420 medium (JRH Biosciences, Lenexa, KS) with equal success. In other published reports on the production of VLPs other media have been reported including:

TC-100, EX-CELL 401, or 405 for production in Tn-5 cells; or TC100, TNM-FH, EX-CELL 400, or 420 for production in Sf-9 cells. See Chapter 8 for a more extensive list of commercially available serum-free media.

4. The advantage of changing medium prior to infection is to insure that limiting nutrients are replenished and inhibitory compounds are eliminated from the cell culture during the production phase; however, there is 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 are increased.
5. Some investigators have used agitation speed as a means to control the dissolved oxygen concentration and have found no adverse effect on the cells. Furthermore, no significant damage at increasing agitation rates were observed in Sf-9 cell culture bioreactors until bubble incorporation occurred because of either cavitation or through vortexing.
6. The moment of harvest depends on how downstream processing is envisaged. If it is desired to keep the AAV vector associated with the cell pellet (this approach will reduce the total amount of AAV production), then the harvest should be done at the latest at 72 h pi. However, by leaving the culture up to 96 h pi, the process is more robust with respect to the initial MOIs of each baculovirus used.
7. To make sure there is no damage to the cells, cells are resuspended in 0.5 mL of PBS followed by the dropwise addition of 0.5 mL of 4% paraformaldehyde solution while vortexing gently.
8. If a fluorescent cell sorter is not available, then a fluorescent microscope can be used to quantify the number of fluorescent cells.

## Acknowledgments

The authors thank Dr. Robert M. Kotin of the National Institutes of Health for supplying the baculoviruses; the National Science and Engineering Research Council and the National Research Council for funding this research in the form of Post-Graduate Scholarships to MGA; the insect cell group whose expertise served as a foundation for this work, specifically Barbara Jardin and Cynthia Elias; Gilles St-Laurent and Yves Durocher for their advice in setting the biochemical methods; and the help of Lucie Bourget whose expertise in flow cytometry has greatly helped move this project forward.

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## Transforming Lepidopteran Insect Cells for Continuous Recombinant Protein Expression

Robert L. Harrison and Donald L. Jarvis

### Summary

The baculovirus expression vector system is widely used to produce large quantities of recombinant proteins. However, yields of extracellular and membrane-bound proteins obtained with this system often are very low, possibly because of the adverse effects of baculovirus infection on the host insect cell secretory pathway. An alternative approach to producing poorly expressed proteins is to transform 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 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 baculovirus expression vector system (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 (*1-3*). Because the polyhedrin protein is not required for infection and replication, recombinant baculoviruses can 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 hundreds of milligrams per liter of baculovirus-infected cell cultures. In addition, baculovirus-infected insect cells and larvae can perform most of the co- and posttranslational protein modifications that occur in mammalian cells, such as proteolytic processing, *N*- and *O*-glycosylation, phosphorylation, acylation, *N*-terminal acetylation, *C*-terminal methylation, and  $\alpha$ -amidation (*see* Chapter 17). The capacity of the BEVS to produce large quantities of recombinant proteins bearing eukaryotic modifications is among its chief advantages.

One chief 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 (4). 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 (5). The promoter used to develop this approach was derived from the baculovirus immediate early gene, *ie-1* (6,7). 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 *Escherichia coli*  $\beta$ -galactosidase under the control of the *ie1* promoter produced far less  $\beta$ -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  $\beta$ -galactosidase, a cytoplasmic protein, can be produced at very high levels with 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 (5). Although the baculovirus-infected cells produced more total (intracellular and extracellular) t-PA, most of the t-PA produced using this conventional system failed to be 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 (8). The results of these subsequent studies have confirmed the trends originally observed by Jarvis et al. and also have 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 previously described has been reproduced and extended by the development of new expression constructs (9–12), by the use of other selection markers (10,13), and by the use of other lepidopteran insect cell lines (12,14).

## 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 (15), have been widely used for this purpose (5,9–11), as has a host promoter derived from a *Bombyx mori* (silkworm) actin gene (12). Baculovirus-derived enhancer elements, known as homologous DNA regions (*hrs*), have been used to stimulate IE-1-mediated transcription in stably transformed insect cells (9). 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 (16).

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, it is necessary to 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 (11) or on a separate plasmid (5). 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) (5), hygromycin B (13), Zeocin™ (10), and blasticidin S (17). 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 previously listed (18). Once selected, indi-

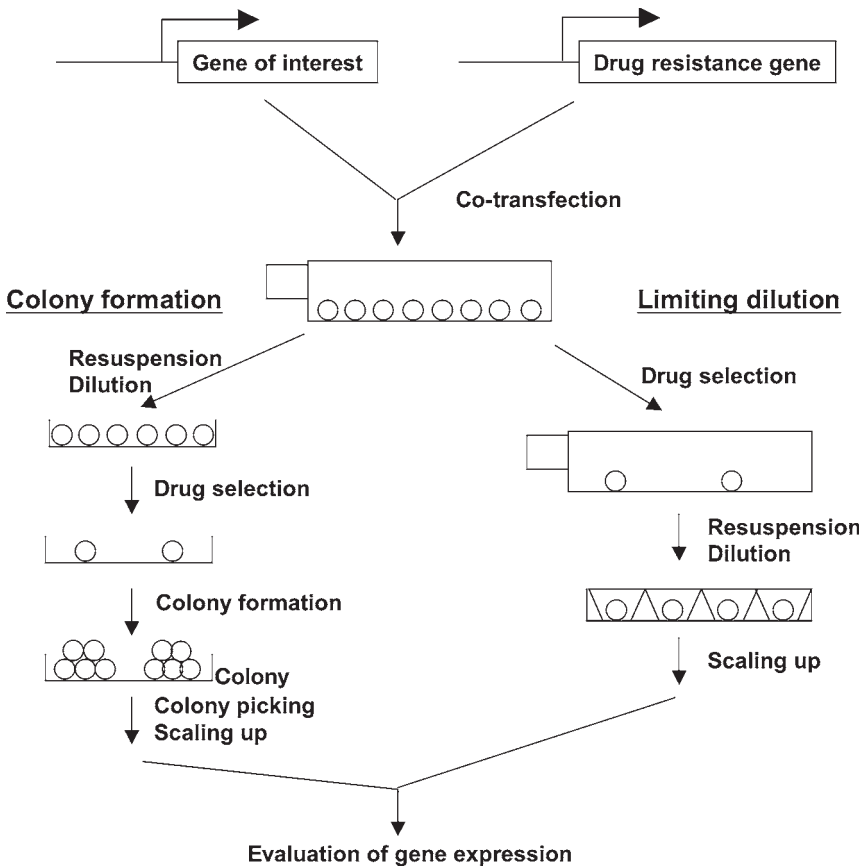


Fig. 1. An overview of two methods for generating monoclonal isolates of transformed lepidopteran cells. Cells are cotransfected with IE1 expression plasmids encoding the gene(s) of interest and a selectable drug resistance marker. After drug selection, clonally derived cell lines can be isolated either 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.

vidual cells can be isolated and expanded to obtain transformed clones. Two methods that the authors have used to obtain clonal transformed insect cell derivatives are detailed next 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 typi-

cally exhibit a wide range of expression levels. Finally, once transformed clones expressing the gene of interest have been identified, it is important to recognize that this is not necessarily a stable genetic trait. 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, although 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.

## 2. Materials

1. Cell lines: Sf-9 (American Type Culture Collection, Manassas, VA, [www.atcc.org](http://www.atcc.org); cat. no. CRL-1711; *see Note 2*) can be obtained from the American Type Culture Collection or from Invitrogen (Carlsbad, CA, [www.invitrogen.com](http://www.invitrogen.com)).
2. Cell media and plastics: Grace's insect medium, Hink's TNM-FH medium, and Pluronic® F-68 are available from JRH Biosciences (Lenexa, KS, [www.jrhbio.com](http://www.jrhbio.com); *see Note 3*). Falcon and Corning flasks, Petri dishes, and multiwell plates for cell culture are available from Fisher Scientific (Pittsburgh, PA, [www.fisher-sci.com](http://www.fisher-sci.com)), as are hemacytometers.
3. Fetal bovine serum is available from Invitrogen (*see Note 4*).
4. Conventional cell culture antibiotics: amphotericin B (Fungizone®) and gentamicin can be obtained from Invitrogen.
5. Antibiotics for selection of transformed cells: G418 (Geneticin®), hygromycin B, Zeocin, and blasticidin S are available from Invitrogen.
6. Enzymes for constructing expression plasmids: restriction endonucleases, calf intestinal alkaline phosphatase, DNA polymerase I (Klenow fragment), and T4 DNA polymerase can be obtained from New England Biolabs (Beverly MA, [www.neb.com](http://www.neb.com)).
7. Plasmids: the pIEHR series of expression vectors (**Fig. 2**), pDIE1HR1 (Chapter 17, **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-iel* enhancer-promoter arrangement (the pIEx series) is available for purchase from Novagen/EMD Biosciences (Madison, WI, [www.emdbiosciences.com](http://www.emdbiosciences.com)). In addition, Invitrogen sells expression plasmids (the pIB and pIZ series) that carry drug resistance genes for blasticidin S and Zeocin.
8. Chemicals: buffers (HEPES, Tris), NaCl, CaCl<sub>2</sub>, EDTA, glucose, hydrochloric acid, NaOH, sodium dodecyl sulfate, sodium acetate, potassium acetate, 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.

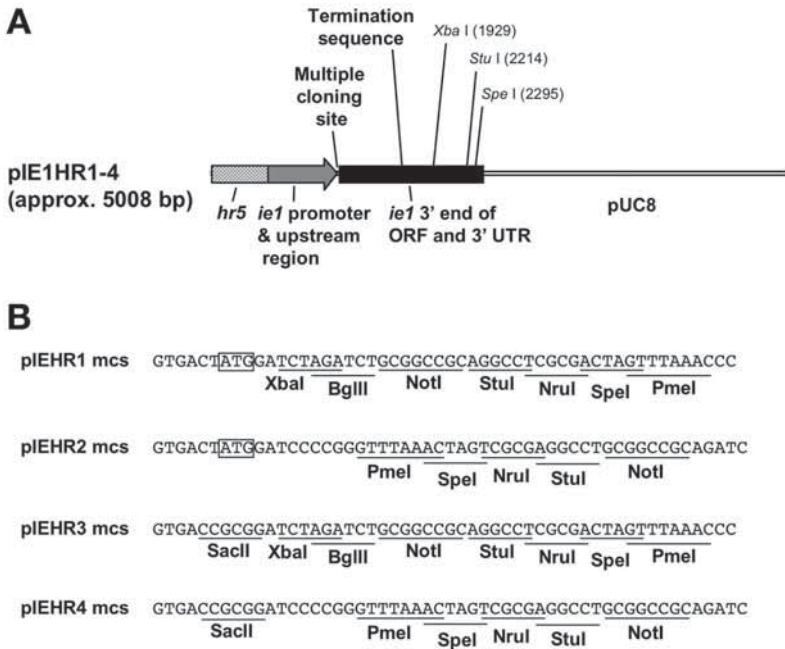


Fig. 2. (A) Features of the pIE1HR series of plasmids used for transformation and constitutive gene expression in lepidopteran cells. The *hr5* enhancer, *ie1* promoter and termination sequence, and multiple cloning site are indicated. Restriction sites that are also present in the multiple cloning sites 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 *SacII* site.

9. Cloning reagents: competent *E. coli* are available from Promega (Madison, WI, www.promega.com). Bacteria media (LB broth, 2xYT), LB agar, ampicillin, and 100-mm plastic Petri dishes for agar plates are available from Fisher Scientific.
10. 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. The authors 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; [9]). This enhancer stimulates IE-1-mediated transcription in transfected or transformed insect cells when these cells are infected with a baculovirus or cotransfected with a plasmid encoding the baculovirus IE-1 protein (6,19). 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 consideration of the open reading frame. We have also constructed a plasmid capable of expressing two genes at once. This “dual” immediate early expression plasmid consists of a single copy of *hr5* flanked by two copies of the IE-1 promoter oriented in opposite directions ([20,21]; see also Chapter 17, Fig. 1). 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 previously described have any selectable markers. Thus, to isolate transformed insect cells, we routinely cotransfect with a separate selection plasmid, which encodes a neomycin- (pIE1Neo; [5]) or hygromycin B- (pIE1Hygro; [13]) resistance marker. The following is an outline of the steps the authors 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 for insertion of 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 *SacII*).
3. Bring the vector preparation volume to 500  $\mu$ L with distilled water. Add 500  $\mu$ L 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 (12,500g for 5 min). Transfer the aqueous (top) phase to a fresh tube and add 50  $\mu$ L 3 M sodium acetate (pH 5.2) and 1 mL 100% ethanol. Vortex briefly and pellet precipitated DNA by microcentrifugation (14,000g 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. Setup 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  $\mu\text{g}/\text{mL}$  ampicillin and incubate plates overnight at 37°C to allow for colony growth.
7. Set up test tubes containing 2.5 mL of 2X YT medium with 50  $\mu\text{g}/\text{mL}$  ampicillin. Inoculate each tube with a colony. Shake tubes at 225 rpm for 8 h to overnight at 37°C.
8. Pipet 1.5 mL of each culture into individual microfuge tubes. Pellet bacteria by brief microcentrifugation (14,000g 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 (22). To start, add 100  $\mu\text{L}$  solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Vortex to resuspend the pellets.
10. Add 200  $\mu\text{L}$  solution II (1.0% sodium dodecyl sulfate, 0.2 N NaOH) to each tube. Mix by inversion. The contents of the tubes should be viscous.
11. Add 150  $\mu\text{L}$  solution III (3 M potassium acetate, 1.8 M formic acid) and mix by inversion. A white flocculent precipitant should appear.
12. Pellet the precipitant by microcentrifugation (14,000g 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  $\mu\text{L}$  deionized, distilled H<sub>2</sub>O. 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 2X 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 (5000g 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 (10,000g 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 (5000g 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 (10 mM Tris-HCl pH 8.0, 1 mM EDTA) appropriate for the type of tube to be used for ultracentrifugation. Add 1.01 g CsCl per gram of suspended pellet and 100  $\mu\text{L}$  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 (23). 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<sub>2</sub>O, 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 mapping.

### 3.2. Transfection

As outlined in **Fig. 1**, cells are cotransfected 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 cotransfection of Sf-9 cells using the calcium phosphate precipitation method (24) prior to antibiotic selection. An alternative method is cationic lipid-mediated transfection with a commercial reagent (such as Cellfectin® from Invitrogen), which should be done following the vendor's recommendations.

1. Seed each of two 25-cm<sup>2</sup> 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 (25 mM HEPES, pH 7.1, 140 mM NaCl, and 125 mM CaCl<sub>2</sub>; *see Note 6*), to the appropriate flasks. Rock the flasks 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 (approx 1 mo). 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 pipet 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. Setup 60-mm Petri dishes with 3 mL complete TNM-FH. Plate diluted cells using the split ratios given next, 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 wk. After 3–4 d, 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 wk 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  $\mu$ L 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 d. 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<sup>2</sup> flask. Allow the cells to grow close to confluency at each step.
13. When the 25-cm<sup>2</sup> flask culture is nearly confluent, split it 1:3. Use two of the resulting 25-cm<sup>2</sup> flasks, which the authors 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 medium from the flasks from **step 8** of the cotransfection procedure (**Subheading 3.2.**) and add 5 mL complete TNM-FH plus selection antibiotic.
2. Incubate flasks 1 wk at 28°C. All the cells in the control flasks should be dead. Many cells in the flasks cotransfected 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 [*see* Chapter 1] culture).
5. Dispense 200  $\mu$ L 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 **Subheading 3.3., step 12.**
7. Process the 25-cm<sup>2</sup> 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 (25) will reveal whether or not the gene is transcribed, whereas Northern blotting (26), S1 nuclease (27), primer extension (28), or RNase protection assays (29) can be used to examine transcription of the gene of interest in more detail. If an antibody is available against the protein of interest, then many standard techniques, such as indirect immunofluorescence (30), immunoprecipitation (4), and Western blotting (31), can be used to detect the gene product in transformed cells. These techniques can also 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 previously mentioned, it should be recognized that not every transformed insect cell clone is 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 recent comparative study of  $\beta$ 1,3-*N*-acetylglucosaminyltransferase 2 ( $\beta$ 3GnT2) expression in baculovirus vector-infected Tn-5 cells and Tn-5 cells stably transformed with the  $\beta$ 3GnT2 gene (32) revealed the same trends observed in 1990 by Jarvis et al. (5). (Note that Tn-5 is the *Trichoplusia ni* BTI Tn-5B1-4 cell line [39] that is commercially available from Invitrogen as High Five™ cells.) Approximately 95% of the  $\beta$ 3GnT2 protein accumulated in cells infected with a conventional baculovirus vector, whereas 90% of the  $\beta$ 3GnT2 protein produced by a transformed cell line was secreted. The extracellular fraction from the transformed cell line contained 2.8-fold more active  $\beta$ 3GnT2 than the same fraction from infected Tn-5 cells and the specific activity of  $\beta$ 3GnT2 purified from the former was 1.6- to 2.9-fold higher than the  $\beta$ 3GnT2 purified from the latter. As

with transformed Sf-9 cells, the transformed Tn-5 cells continuously expressed the gene of interest for over 100 passages in culture.

Other advantages of expression from stably transformed cells have been reported:

- a. Baculoviruses produce a cysteine protease during infection that can degrade foreign proteins and further reduce the yield of secreted protein (33,34). The study previously cited (32) showed that the  $\beta$ 3GnT2 produced with a baculovirus expression vector was extensively degraded, whereas there was no degradation of this enzyme when it was produced in stably transformed Tn-5 cells.
- b. Patch clamp analysis of Sf-9 cells expressing the bovine GABA<sub>A</sub> receptor was significantly easier to perform on cells stably transformed with the GABA<sub>A</sub> receptor gene than on cells infected with a GABA<sub>A</sub> receptor-expressing baculovirus (35).
- c. Human  $\beta$ 2-adrenergic receptor expressed in stably transformed Sf-9 cells was able to functionally couple with the Sf-9 cell endogenous adenylyl cyclase system, something not observed when the  $\beta$ 2-adrenergic receptor was expressed using a standard baculovirus vector (36).

The results of these studies suggest, in general, that the use of stably transformed insect cells for foreign protein production can circumvent many difficulties associated with the conventional baculovirus-infected cell approach, particularly for functional analyses of secretory pathway proteins.

2. For transformations, we usually start with the Sf-9 subclone of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE (37). 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 (38). 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  $\mu$ g/mL amphotericin B and 25  $\mu$ g/mL gentamycin were highly resistant to neomycin selection (18). We also have successfully transformed Tn-5 cells (39) using the procedures described herein, though we were unable to isolate single cell clones by limiting dilution in that particular study (20).
3. Grace's and TNM-FH media also can be prepared according to published recipes (24). 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 medium because studies performed at HyClone have shown that heat inactivation has no positive influence on insect cell culture (40). 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 to 20  $\mu$ g of expression plasmid, there does not appear to be any significant difference in the transformation efficiencies or expression levels obtained from 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 **Subheading 3.3., step 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 (1) determine if the cells were previously exposed to antibiotics and (2) 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 ultraviolet 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 in clumps 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<sup>2</sup> flask surface, pelleting the cells by low-speed centrifugation for 1 min, and resuspending in complete TNM-FH plus 10% DMSO. Optimal cell cryopreservation and recovery is achieved with a cooling rate of  $-1^{\circ}\text{C}/\text{min}$ . The Cryo  $1^{\circ}\text{C}$  Freezing Container ("Mr. Frosty") from Nalgene (Rochester, NY; cat. no. 5100-0001) provides this cooling rate. Subsequently, the cells should be placed in liquid nitrogen for long-term storage.

## Acknowledgments

DLJ gratefully acknowledges the National Institutes of Health (GM49734), the National Science Foundation (BES-9814157 and BES-9818001), and the US Department of Agriculture-National Research Initiative (89-37266-4935 and 95-37302-1921/2658) for supporting work in his lab. 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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## Transformation of *Drosophila* Cell Lines

*An Alternative Approach to Exogenous Protein Expression*

Lucy Cherbas and Peter Cherbas

### Summary

Techniques and experimental applications are described for exogenous protein expression in *Drosophila* cell lines. Ways in which the *Drosophila* cell lines and the baculovirus expression vector system differ in their applications are emphasized.

**Key Words:** *Drosophila*; S2; Kc; stable transformation; transient expression.

## 1. Introduction

### 1.1. *Drosophila* Cell Lines as an Alternative Expression System

Transformed *Drosophila* cell lines have properties that make them quite attractive for expressing exogenous proteins. Two lines (S2 and Kc) have been widely used in the *Drosophila* community since techniques for their transfection were established in the early 1980s. They have proved extremely useful for a wide variety of experiments, including large-scale exogenous protein expression. In recent years, S2 cells have become popular outside the *Drosophila* community as well, as an alternative to the baculovirus expression vector system (BEVS) for high-level exogenous protein expression. This increased popularity of S2 cells is directly attributable to an aggressive advertising campaign for Invitrogen's "Drosophila Expression System" kit. The Invitrogen system is based on previously established techniques, with the addition of a series of useful vectors designed for targeting expression products for secretion and for adding epitope tags to the expressed protein. Invitrogen has also added a new selection system.

### 1.1.1. Critical Properties That Make These Lines so Useful

1. Transformation routinely leads to the incorporation of hundreds or thousands of copies of the exogenous plasmid DNA (1), thereby making high-level expression relatively easy to achieve. Plasmids transfected into S2 cells recombine by homologous recombination to form long tandem arrays. When multiple plasmids are transfected, they recombine through common sequences (usually vector sequences) to form mixed arrays that contain all of the plasmids in approximately the same proportion as the input mixture (2). The incorporation of these large arrays into the chromosome makes it easy to achieve high expression levels in transformed cells, and the formation of mixed arrays makes it possible to provide the expression construct and the selectable marker in separate plasmids and still be confident that cells selected for the presence of one plasmid will contain both of the input plasmids (2). The technique was originally used with two plasmids—a selection plasmid and an expression plasmid, but it can be expanded to multiple plasmids, making possible the simultaneous expression of multiple transgenes (3). The formation of very large tandem arrays following transformation seems to be a common characteristic of dipteran cell lines; mosquito cell lines incorporate arrays containing 10,000 or more copies (4). It was recognized quite early that this property of transformed fly cells could be used to generate high exogenous protein expression levels. As a proof of principle, Moss (2) established clones of transformed S2 cells in which about 1% of the poly(A)<sup>+</sup> RNA was derived from an exogenous *Drosophila* *Adh* transcription unit. The endogenous *Adh* gene is not expressed in S2 cells, but the high level of expression from the transgene did not appear to damage the cells. To our knowledge, the first published cases in which these approaches was put to practical use were the generation of an appropriately glycosylated HIV envelope protein for biochemical studies (5) and the generation of the *Drosophila* protein Wingless for studies of its signaling properties (6).
2. The transformation of *Drosophila* cells is non-lytic, in contrast to the BEVS. Therefore, stably transformed *Drosophila* cells can provide a permanent, stable source of an engineered protein. In addition, the presence of a plasmid-encoded protein in an otherwise normal cell permits the examination of its properties *in situ*. For experiments of this type, the choice of cell line may be important; for example, our laboratory has used Kc cells for many years to study the mechanism of ecdysone action, because Kc cells have a much more robust response to the hormone than do S2 cells. S2 or Kc cells, transfected either transiently or stably are used for a wide variety of experiments, of which the following are a few examples:
  - a. Cells may be transformed to express individual cell-surface proteins (wild type or mutated) that are not normally expressed in the cell line, and populations of cells transformed to express individual proteins may be mixed to examine the interaction of these proteins in a situation that approximates

their normal context. For example, mixtures of transformed S2 cells expressing fasciclin I or fasciclin III were used to demonstrate that these cell adhesion molecules formed exclusively homotypic complexes (7,8). Similarly, S2 clones expressing Delta, Notch, or Serrate from transgenes permitted studies of homo- and heterotypic interactions of these signaling proteins in vitro (9,10).

- b. The cytoskeleton may be perturbed by the expression of a component not normally expressed, or labeled by the expression of a fluorescent version of a protein that is normally present in the cell. A role for neuroglian in organizing the cytoskeleton was demonstrated by the observation that exogenous expression of neuroglian in S2 cells leads to a cell-adhesion-dependent ankyrin localization (11). GFP-tagged actin, tubulin, and kinesins were expressed in S2 cells to study the properties of these cytoskeletal components in living S2 cells (12–14).
  - c. Transcriptional regulators have been studied extensively in cells transfected with plasmids expressing transcription factors (wild type or mutant) and appropriate promoter-reporter constructs. The role of hox gene products as transcriptional regulators was demonstrated in S2 cells (15,16). The transcriptional activation domains of the ecdysone receptor were mapped in transfected Kc cells (17). The same approach was used to study mammalian Sp-family transcription factors, taking advantage of the fact that S2 cells, unlike the standard mammalian cell lines, have no endogenous Sp transcription factors (18). The utility of stable transformation for studying transcriptional regulation is limited by interactions within a long array of plasmid sequences; for these experiments, transient transfections are usually preferable.
  - d. Stably transformed S2 cells that express individual receptors and ion channels of both *Drosophila* and mammalian origin have been valuable tools for studies of these membrane components (19).
  - e. The ability to knock out specific gene expression easily in *Drosophila* cell cultures by use of RNAi (20,21) has vastly expanded the possibilities for studying the function of engineered transgenes in transfected cells (e.g., see refs. 14, 22, and 23).
3. Processing of RNA and proteins is reasonably close to that seen in mammalian cells. In this respect, *Drosophila* cells resemble the BEVS and are preferable to bacterial or yeast systems. The relative fidelity of processing in S2 and in the BEVS (with Sf-9 host cells) varies, as illustrated in the following examples:
    - a. The catalytic domain of ADAM33, a human pro-metallopeptidase associated with asthma susceptibility, was expressed at high level and correctly processed in S2 cells, but processing in the BEVS was poor (24).
    - b. Cobra venom factor is processed in a series of three proteolytic steps. When the protein was expressed in *E. coli* no processing took place and the protein was inactive. The first two processing steps occurred in S2 cells and more slowly in Sf-9 cells, and the product was biologically active (25,26).

- c. Human cyclooxygenase-2 has a complex glycosylation pattern; S2 cells replicated the mammalian pattern more closely than Sf-9, but expression in Sf-9 cells was several orders of magnitude higher than in S2 cells (27).
- d. Mouse and ST2/T1 and a human interleukin receptor all were less heavily glycosylated in S2 than in mammalian COS cells (28); grasshopper fasciclin I was less heavily glycosylated in S2 than in mammalian CHO cells (29).
- e. A small peptide spider toxin with multiple sulfhydryl bridges proved difficult to express in active form in a bacterial expression system, but was produced in a correctly folded, active form in S2 cells (30).

## 1.2. Available Cell Lines

S2 cells have been used almost exclusively among *Drosophila* cell lines for preparative expression of exogenous proteins. The line is easy to grow, and its transformation properties are relatively well characterized. S2 cells grow loosely adherent to the substrate, and large-scale cultures can easily be grown in spinner flasks. Kc cells are equally easy to grow, have similar transformation properties, and are less expensive to maintain because they require less serum; their relative lack of popularity is an historical accident.

A large number of additional *Drosophila* cell lines have recently become available, including lines from different tissue sources. S2 and Kc were made from *Drosophila* embryos; their properties suggest a hemocyte origin for both lines (31,32). There are now a few dozen additional embryonic lines, as well as lines derived from imaginal discs (33,34) and from the larval central nervous system (35). S2 and Kc remain the lines of choice for expression of exogenous genes, because they are well characterized and easy to handle. It is quite possible, though, that lines from different tissue origins may prove to be more appropriate in some cases, because the processing of a protein often depends on the tissue from which a cell line is derived as well as its species. An example in point is the properties of membrane receptors (LDL receptor, transferrin receptor, and lipophorin receptor), whose ability to recycle their ligands appear to be similar in fat tissue from mammals and insects, but not in S2 or Sf-9 cells (36). Thus, although none of the disc or central nervous system lines have yet been used as an expression system, they have the potential to be helpful in the future. In particular, because these lines retain many of the properties of the tissues from which they were derived, they may prove useful for examining the function of labeled or mutated proteins *in situ* in different developmental contexts.

## 1.3. Types of Transformation

### 1.3.1. Cotransformation of Selection and Expression Plasmids

In most transformation systems, when one wants to engineer the expression of a protein, it is necessary to construct a plasmid containing both the desired

promoter and coding sequence and either a selectable marker or the required virus sequences. A few workers have used this approach for transforming *Drosophila* cells (37,38), but the ability of S2 or Kc cells to recombine plasmids in long arrays makes it possible to use cotransfection of multiple plasmids instead (see **Subheading 1.1.1.**). The cells are simply transfected with a mixture of plasmids, one carrying the selectable marker and another carrying the expression construct. Not only does this procedure avoid the difficulties of constructing very large plasmids, but it also permits one to vary the ratio of the two plasmids in the mixture and thereby vary their ratio in the transformed cell. In this way, one can control to some extent the amount of the expression plasmid incorporated into the cell and thereby the level of expression of the transgene. Varying the amount of the expression plasmid may be quite useful, because many exogenous proteins are toxic to the host cell, killing transformants with high levels of expression (see **Note 1**). Even when one uses an inducible promoter, the basal (uninduced) expression level may be sufficient to prevent the recovery of transformants.

The fate of transformed plasmids has been studied in detail only in S2 cells transfected using a calcium phosphate–DNA coprecipitate (2). These cells typically take up about 1000 copies of plasmid per haploid genome (i.e., 4000 copies per cell). The formation of arrays is not driven by selection, because the selectable marker used in these experiments (pHGCO, which confers resistance to methotrexate) gave full protection at five copies per haploid genome. Moss (2) found that the ratio of plasmids in the transformed cells was approximately equal to the ratio of plasmids in the transfection mixture, and therefore even when the expression plasmid was only 1% of the input mixture, all methotrexate-resistant cells contained the expression plasmid. To achieve a range of levels of expression, he found it useful to transfect with mixtures varying between 1:1 and 1:100 (expression plasmid to selection plasmid).

S2 cells can be transfected by a variety of protocols; no data are available on the array structure following electroporation or lipofection, but anecdotal evidence from the literature and from our laboratory suggests that these procedures also lead to the formation of mixed arrays. The data for transformed Kc cells are even less complete, but here, too, anecdotal information suggests that the results are similar to those seen in S2 cells.

### 1.3.2. P Element Transposition

An alternative approach to transformation of *Drosophila* cells is the use of P element transformation (39). A plasmid containing the expression construct and the selectable marker, flanked by a single pair of P element terminal repeats, is transfected into the cells along with a small amount of a helper plasmid, which expresses P transposase. The P element, containing both expression construct

and selectable marker, inserts as single copies at random sites in the chromosome; the number of inserts can be regulated to some extent by varying the amount of plasmid used for the transfection (*see Note 2*). If a suitable selectable marker is used for which a single copy is sufficient to confer resistance to the selective agent, then one can recover transformed clones that have a single copy of the expression plasmid. This procedure is of potential value in cases where it is necessary to restrict expression to a low level, or when it is desirable to avoid the specialized chromatin structure associated with long tandem arrays.

#### **1.4. Types of Selection and Available Selection Plasmids**

**Table 1** lists five selective agents commonly used for stably transforming *Drosophila* cells. In our laboratory, methotrexate is the selective agent of choice because it is effective and very inexpensive.  $\alpha$ -amanitin is a very effective selective agent (**40**) and has been used successfully for generating S2 cell expression lines (**8,41**), but it has the disadvantages of high price and very high human toxicity. G418 has been used successfully by several workers (**27,29,37,42,43**), but a high rate of spontaneous resistance makes it a relatively poor selective agent (**44**) (Cherbas, L., unpublished observations). Hygromycin B and blasticidin S are also widely used for selection in *Drosophila* cell lines; our laboratory has little or no experience with these agents. Multiple selection systems may be used for sequential transformations, although this is usually unnecessary because multiple plasmids can be incorporated in a single transfection.

#### **1.5. Types of Promoters**

Promoters for expression fall into two broad categories: constitutive and inducible. Examples of each are shown in **Table 2**. Inducible promoters are commonly used in stable transformation to minimize the toxicity that often results from high-level expression of an exogenous protein. Transformed cells are selected under conditions where the promoter is minimally active; the promoter is induced to high-level expression for a short time immediately before harvesting. The most commonly used inducible promoter for expression in *Drosophila* cell lines is derived from the *Drosophila* metallothionein (Mt) gene. This metal ion-induced promoter was first developed for use in S2 cells by Bunch et al. (**45–47**), and subsequently popularized by the Smith/Kline group (**44,48,49**) and Invitrogen. It typically gives about a 30-fold induction upon treatment with 1 mM CuSO<sub>4</sub> (**46**), but both the basal expression level and the induction vary widely among transformed clones (**[2,50]**; Cherbas, L., unpublished observations). Cloning of transformed cells (*see Subheading 3.4.2.*) permits one to select clones with mini-

**Table 1**  
**Selection Systems**

Selective agent	Concentration used	Plasmid
Methotrexate	$2 \times 10^{-7}$ M	pHGCO (1) p8HCO (10) actDHFR (39)
$\alpha$ -amanitin	5 $\mu$ g/mL for S2; 10 $\mu$ g/mL for Kc	pPC4 (40)
Hygromycin	Approx 200–300 $\mu$ g/mL (Invitrogen manual)	pcophygro (38) pCO*hygro (44) pBKS-Ubi-hygro (64) pUC-HygroMT (65)
G418	pcopneo (19) pCO*neo (44)	
Blastocidin	Approx 5 $\mu$ g/mL (Invitrogen manual)	PCoBlast

mal basal expression and maximal induction ratio.  $\text{Cu}^{2+}$  is usually used for induction of the Mt promoter; it is not the most potent inducer, but it is less toxic to the cells than other divalent metal ions. However, in at least one case, the quality of the expressed protein depended on the nature of the ion used for induction of the promoter:  $\text{Cd}^{2+}$  induction of a Mt-ADAM33 expression construct gave a higher yield and quality of the product than  $\text{Cu}^{2+}$  induction (24). Even 1 mM  $\text{CuSO}_4$ , the concentration normally used for induction of the Mt promoter, has significant effects on the physiology of S2 and Kc cells; this must be taken into consideration in interpreting experiments that analyze the function of transgenes in the cells. A heat-inducible hsp70 promoter has also been used for induction of expression in S2 cells (6). In our experience, the hsp70 promoter has a basal expression level comparable to the metallothionein promoter and an induction ratio generally lower than that of the Mt promoter (because the strong transcriptional induction is balanced by a heat-shock repression of translation), and heat-shock has even more profound physiological effects than  $\text{CuSO}_4$ ; we therefore do not recommend it as an alternative to the Mt promoter. A progesterone-inducible promoter is used for expression in flies (51), but to our knowledge it has not yet been tested in cell culture. Because all inducible promoters have some basal level of expression, it may be impossible to produce a stable line with satisfactory expression of some proteins that are severely toxic to the cell. Under these circumstances, the recovery of transformants is very low, and those trans-

**Table 2**  
**Promoters**

Promoter	Type of expression	Plasmid	Public source	Comments
Actin5C	Strong, constitutive	pPac (15)		
		RAct-HAdh (Cherbas, L., unpublished)	DGRC	
		pAc5.1/V5-His	Invitrogen	C-terminal epitope tag and His6
		pAW (66)	DGRC	Gateway™ cloning technology
		pCMA (17)	DGRC	Includes promoters for expression in mammalian cells and in vitro
		pAGW, pAWG, pACW, pAWC, pAVW, pAWV, pAHW, pAWH, pAMW, pAWM, pAFW, pAWF, pAFHW, pAFMW, pARW, pAWR, (66)	DGRC	Gateway cloning technology, variety of epitope and fluorescence tags
Tubulin	Strong, constitutive	No published vectors		Used for Wg expression in S2 (67)
Ubiquitin	Strong, constitutive	No published vectors		Used by various laboratories, none published
Metallothionein	Inducible	pRmHa1, pRmHa2, pRmHa3 (45,46)	some in DGRC	
		pMt/V5-His	Invitrogen	C-terminal epitope tag and His6
		pMt/V5-His-TOPO	Invitrogen	TOPO™ cloning technology, C-terminal epitope tag, and His6
		pMt/DEST48	Invitrogen	Gateway cloning technology, C-terminal epitope tag, and His6

formed cells that are recovered often have lost the expression plasmid altogether; if the expression plasmid is present, then it is usually mutated in some fashion ([2]; Cherbas, L., unpublished observations).

Occasionally, high-level expression of a transgene is well tolerated in a stably transformed line, permitting the use of a strong constitutive promoter. For example, the Nusse laboratory substituted a strong constitutive tubulin promoter for the heat-shock promoter initially used for Wingless expression in S2 cells (R. Nusse, personal communication); the resulting line is quite healthy, and the constitutive expression of Wingless makes the cells much more convenient to use. We have also found that Kc cells expressing high levels of specific EcR (ecdysone receptor component) isoforms from an actin promoter are healthy, though their response to ecdysone is abnormal (Cher, L., unpublished observations). In transient expression experiments, there is no need to keep the transformed cells alive for an extended period, and a strong constitutive promoter is usually the preferred means of expressing exogenous proteins. For strong constitutive expression, the *Drosophila* actin5C promoter is commonly used. This promoter was first used for expression in *Drosophila* cell lines in the Hogness lab (15,16).

## 2. Materials

### 2.1. Cell Lines (see Note 3)

1. S2 (*Drosophila* Genomics Resources Center, DGRC [<http://dgrc.cgb.indiana.edu/>]; American Type Culture Collection [<http://atcc.org/>]; Invitrogen).
2. Other cell lines (*Drosophila* Genomics Resources Center [<http://dgrc.cgb.indiana.edu/>]).

### 2.2. Media and Equipment for Cell Culture

1. Schneider's medium (52) (available in powdered or liquid form) (Sigma, Invitrogen).
2. M3 medium (53) (available in powdered or liquid form) (Sigma).
3. M3+BPYE: M3 supplemented with bactopectone (DIFCO, 2.5 g/L final concentration) and yeast extract (Sigma, 1 g/L final concentration; this is in addition to the 1 g/L contained in M3). Use tissue-culture grade yeast extract, not a yeast extract that is sold for bacterial medium.
4. CCM-3 (available in powdered or liquid form) (HyClone).
5. Freezing medium: normal growth medium (including serum) supplemented with 10% dimethylsulfoxide.
6. Fetal calf serum (HyClone, Sigma, various other suppliers), heat-treated for 30–60 min at 56°C (see Notes 4 and 5). We recommend a high-grade serum. We store serum at –20°C for up to several years prior to heat-treatment, and at 4°C for up to a few months following heat-treatment.

7. Tissue culture-grade plastic ware (Petri plates, T-flasks, multiwell flasks) (any standard laboratory supplier).
8. Wide-mouth Dewar flask (VWR, other laboratory supply houses).

### 2.3. Expression Vectors (see [Table 2](#))

1. Metallothionein promoter vectors for inducible expression (DGRC, Invitrogen) (see [Note 6](#)).
2. Actin promoter vectors for strong constitutive expression (DGRC, Invitrogen) (see [Note 7](#)).

### 2.4. Selection Vectors

1. Methotrexate selection vectors (pHGCO, pHCO [[1](#)], p8HCO [[10](#)], actDHFR [[39](#)], p8HCO, and actDHFR are available from DGRC) (see [Note 8](#)).
2. Hygromycin selection vectors (Invitrogen) (see [Note 9](#)).
3. Blastomycin selection vectors (Invitrogen).

### 2.5. Calcium Phosphate–DNA Coprecipitation

1. 2X BES-buffered saline (2X BBS): 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM BES, pH 6.95, filter-sterilized and stored at –20°C (see [Note 10](#)).
2. 2 M CaCl<sub>2</sub>, filter-sterilized and stored at –20°C.
3. Plasmid DNA dissolved in TE and stored at –20°C. In general, increasing DNA purity leads to increased reliability and reproducibility of the transfection. We routinely purify plasmids on a continuous CsCl gradient ([54](#)); other standard purification procedures are commonly used for preparation of DNA for transfection and usually give acceptable results. DNA usually does not require sterilization, but if it seems to be a source of contamination, it can be sterilized by overnight precipitation in 70% ethanol.

### 2.6. Electroporation

1. A power supply suitable for electroporation of eukaryotic cells. We use a Hoefer PG200 Progenitor with PG250 electroporation chamber and PG220C cuvet electrode; this equipment is no longer made, and the protocol must be adapted to the properties of the equipment available.

### 2.7. Lipid-Based Transfection Techniques

A large number of lipofection and other lipid-based kits are commercially available. Some systems that we have used successfully in our laboratory are listed here.

1. FuGene 6 (Roche Applied Science).
2. Cellfectin (Invitrogen).
3. Effectene (Qiagen).

## 2.8. Selective Agents

The agents listed next are available from many sources; we have listed a few, but do not wish to imply that these sources are necessarily better than their competitors.

1. Methotrexate (MTX) (Sigma-Aldrich, Fisher). Store the stock solution ( $4 \times 10^{-4}$  M in 10 mM  $\text{Na}_2\text{CO}_2$ ) at  $-20^\circ\text{C}$  protected from light. After dilution in medium, store at  $4^\circ\text{C}$  protected from light for no more than 2 wk (*see Note 11*). The stock solution may be frozen and thawed many times, and is stable for years. Use at a final concentration of  $2 \times 10^{-7}$  M.
2.  $\alpha$ -Amanitin (Sigma-Aldrich, Fisher). Dissolve in  $\text{H}_2\text{O}$  at 1 mg/mL, and store the stock solution at  $-20^\circ\text{C}$ . Use at a final concentration of 5–10  $\mu\text{g}/\text{mL}$ .
3. G418 (American Type Culture Collection, Sigma-Aldrich, Stratagene).
4. Hygromycin B (Calbiochem, Hygromycin.com, Invitrogen, Sigma-Aldrich, Stratagene, and many other sources). Use at a final concentration of approx 200–300  $\mu\text{g}/\text{mL}$  (Invitrogen manual).
5. Blasticidin S (Calbiochem, Invitrogen, Sigma-Aldrich). Use at a final concentration of about 5  $\mu\text{g}/\text{mL}$  (Invitrogen manual).

## 2.9. Preparing a Feeder Layer

1. Cells. Feeder cells are normally derived from cells similar to the cell line that is used for transformation; if possible use a cell line that has been transformed to give resistance to the selective marker that was used for your transformation.
2. Cesium source or X-ray machine capable of delivering 24 kR of radiation to a 50-cm<sup>2</sup> T-flask.
3. Robb's saline (55) (*see Subheading 3.4.1.* for preparation details).

## 2.10. Cloning in Soft Agar

1. 1.5% Noble agar (Difco) in water: autoclave to sterilize, and store at room temperature. Prior to use, melt (by brief autoclaving or by heating in a microwave oven), and store at  $45$ – $50^\circ\text{C}$  (*see Note 12*).
2. 100-mm Bacteriological-grade Petri plates (*see Note 13*).

## 3. Methods

Detailed protocols for growing S2 and Kc cells can be found in previous publications (56–58); herein we provide a brief summary. *Drosophila* cell lines are grown at  $25^\circ\text{C}$  with air as the gas phase. Both S2 and Kc are loosely adherent on the substrate. We use tissue culture-grade Petri plates for routine maintenance (10 mL cell culture per 100-mm plate, with plates kept in sealed food-storage containers to maintain humidity), and spinner flasks for large-scale cultures; T-flasks may also be used, but we find them to be less desirable for routine maintenance because they are more expensive than Petri plates and

the cells are less easily accessible for manipulation. Both lines grow exponentially between about  $5 \times 10^5$  and  $1 \times 10^7$  cells/mL, and under optimal conditions grow with a doubling time of 16–20 h. In general, we recommend diluting the cells 10-fold when they reach  $10^7$  cells/mL, i.e., every 2–3 d. Transformed lines may, of course, grow more slowly, and may have different growth requirements.

### 3.1. Media

Use a medium appropriate for the cell line that you are growing. In our experience, most *Drosophila* lines grow well in Schneider's medium and in M3 + BPYE; both of these media require supplementation with fetal calf serum. Our laboratory uses M3 + BPYE. S2 cells require 10% serum; Kc cells grow equally well with 5% serum. Kc cells (but not S2) can be grown in CCM-3, a serum-free medium sold by HyClone for Sf-9 cells; aside from the fact that the cells adhere slightly more strongly to the substrate in CCM-3, their growth characteristics are identical to those in serum-containing medium, and the cells may be switched back and forth between CCM-3 and the serum-containing medium without any apparent adverse effects.

### 3.2. Storage of Cell Lines (see Note 14)

#### 3.2.1. Freezing Viable Cells

1. Centrifuge a culture of healthy cells from mid-exponential growth (see Chapter 1), and resuspend them at approx  $2 \times 10^7$  cell/mL in freezing medium. For example, resuspend cells from a 10-mL plate at  $5 \times 10^6$  cells/mL in 2.5 mL freezing medium.
2. Dispense the cell suspension into cryo-vials, 0.5 mL per vial, and place the vials into a wide-mouth Dewar flask at room temperature. Seal the flask with a foam stopper or equivalent closure, and use tape to ensure that the stopper does not fall off as the flask is chilled.
3. Place the Dewar flask in an  $-80^\circ\text{C}$  freezer for 1–2 d to allow the cell suspension to freeze slowly.
4. Once the vials have reached  $-80^\circ\text{C}$ , they may be transferred to liquid nitrogen without damaging the cells.

#### 3.2.2. Thawing Frozen Cells

1. Prepare a small (50 cm<sup>2</sup>) T-flask (see Note 15) containing 5 mL of medium appropriate to the cell line.
2. Using a Pasteur pipet, add about 0.5 mL of medium to the ampoule while the cells are still frozen. Pipet up and down to thaw the contents of the ampoule.
3. Add the thawed cells back to the T-flask.
4. To remove the dimethylsulfoxide, either centrifuge the cell suspension and resuspend the pellet in fresh medium, or permit the cells to settle on the surface of the plate and then gently remove the medium and replace it with fresh medium.

### 3.3. Transfection

Calcium phosphate–DNA coprecipitation is the original technique used for transforming fly cells, and it is the only technique for which the resulting stably transformed plasmid arrays have been extensively studied. All of the techniques described next have been used successfully for transient expression, and a variety of laboratories including ours have been successful in using them for stable transformation, but the stable products have not been well characterized in any case except calcium phosphate (*see Note 16*).

#### 3.3.1. Calcium Phosphate

The ability of cell lines to be transformed using a calcium phosphate–DNA coprecipitate is highly variable and unpredictable. S2 cells are easily transformed with this technique, as is one clone of Kc (Kc167), but many other Kc clones and sublines fail to be transformed, as do some other *Drosophila* lines, e.g., S3.

1. Use approx  $3 \times 10^6$  cells/mL in serum-containing medium. (Anecdotal evidence suggests that the calcium phosphate transfection procedure is incompatible with CCM-3 medium.) For stable transformations, use 3 mL cells in a 60-mm plate; for transient expression, use 10 mL cells in a 100-mm plate. The procedure for stable transformation leads to higher transfection efficiency, but the cells are quite unhealthy for a few days, making them unsuitable for transient expression experiments.
2. For each plate to be transfected, prepare 1 mL of precipitate, as follows: in a 15-L sterile plastic tube, mix 62  $\mu$ L 2 M CaCl<sub>2</sub>, 20  $\mu$ g DNA, and H<sub>2</sub>O to 0.5 mL. Agitate the solution by bubbling air through a sterile cotton-plugged Pasteur pipet; at the appropriate flow rate at least two to three bubbles are always in transit. While continuing the aeration, add 0.5 mL 2X BBS dropwise. Remove the pipet, and let the suspension sit for 30 min before using. The precipitate produces a faint bluish tint from light scattering, but there should be no visible settling of the precipitate during this time. If you are transfecting multiple plates with the same plasmid mixture, then scale up the volumes appropriately, and use a 1-mL aliquot for each plate.
3. Add 1 mL of the suspension dropwise to each plate, and mix gently by tipping the plate back and forth. After a few hours, the precipitate will be visible on the bottom of the plate, looking uncomfortably like bacterial contamination. The precipitate can be distinguished from bacteria by the fact that the precipitate is less uniform in size and shape than bacteria, and it fails to increase with time.
4. Wash the cells 3–16 h after the addition of the precipitate by replacing the medium twice with fresh medium. If the cells are sticking well to the substrate, then simply withdraw the medium and add an equal volume of new medium. If there is a significant population of floating cells, then recover them by centrifugation (2 min at 1000g) and add them back to the plate.

5. Allow the cells to recover and express the proteins encoded in the transfected plasmids for 1–2 d. For transient expression, the optimal time for harvesting depends on the stability of the expressed RNA and protein and must be determined for each experimental system. For stable transformation, allow the cells to express the selectable marker for 2 d before adding the selective agent.

### 3.3.2. Electroporation

1. Use a culture set up at least 1 d before the electroporation; the cell density should be approx  $3 \times 10^6$  cells/mL.
2. Centrifuge the cells (2 min at 1000g) and resuspend in medium without serum. Repeat the process, and adjust the volume for the second resuspension so that the volume of cell suspension is appropriate for the number of transfections to be done (see **Note 17**).
3. Dispense cell suspension into electroporation chambers (cuvets). With our equipment, we use 0.8 mL cell suspension per cuvet.
4. Add DNA, mix gently, and incubate at room temperature for 5–10 min. The quantity of DNA used in a transfection may be varied widely; we have used between 1  $\mu$ g and 1 mg per transfection, with the resulting level of expression (by transient expression) or the number of stably transformed clones roughly proportional to the amount of input DNA.
5. Shock the cells at 440 V/cm for Kc cells, 700 V/cm for S2 cells; 120  $\mu$ F; 1 s (i.e., complete decay). These parameters may vary somewhat with different equipment. For other cell lines, it may be necessary to optimize the field strength, but we have found 440–700 V/cm to work for many *Drosophila* lines.
6. Allow cells to recover for about 10 min, and then add the cells to an appropriate volume of medium with sufficient serum to bring the final serum concentration to the level appropriate for the cell line you are using.

### 3.3.3. Lipofection and Other Lipid-Based Systems (see **Note 18**)

Our experience with lipid-based transfection systems is too limited for us to recommend specific systems or to provide specific protocols. Follow the instructions provided with the kit, and bear in mind that conditions must be optimized for each cell line.

## 3.4. Selection

The most effective method for obtaining a stable cell line with the desired expression properties is to clone the transfected population while they are undergoing selection. This offers two significant advantages over bulk selection of a transfected population. First, a clone of transformed cells is more homogeneous and more stable in its expression characteristics than an uncloned popu-

lation of transformed cells (*see Note 19*). Second, there is usually a large variation among clones both in the level of expression of a transgene (whether measured as expression per cell or expression per plasmid copy), and in the induction ratio for an inducible promoter. If the cells are cloned during the selection process before significant competition occurs between transformed cells, then the clones retain much of the original heterogeneity of the transformants. One can then simply choose a clone whose expression properties are best for the experimenter's purposes. Nonetheless, most workers choose to select the population without cloning, because it is significantly easier and faster.

For either procedure, wait 2 d before adding the selective agent to the medium; this delay is intended to allow the cells to express an adequate level of the resistance marker encoded by the selection plasmid that has just been transfected into the cells.

### 3.4.1. Population Selection

1. Allow 2 d after transfection to allow the cells to express the selection marker.
2. Add the selective agent at the recommended concentration (*see Subheading 2.4.* or **Table 1** for guidelines).
3. Replace the medium about twice a week with fresh medium containing the selective agent, until growing cells reach a high enough density that the culture can be expanded. One can often see a few divisions in cells that are expressing the selectable marker transiently but fail to incorporate the plasmids into the chromosome, but usually cells that continue to grow for at least 2 wk are stably transformed.
4. Once the growing cells are at a high enough concentration that the culture can be safely diluted, expand the culture as you would normal, untransformed cells, but if possible, retain the selectable marker in the medium (*see Note 20*).

### 3.4.2. Clonal Selection

#### 3.4.2.1. PREPARATION OF FEEDER CELLS

*Drosophila* cell lines will not grow at all at densities less than about  $10^4$  cells/mL; hence to clone cells from low density it is necessary to mix them with an irradiated feeder layer.

1. Resuspend an appropriate number of cells (the density during irradiation does not seem to be important) in 5 mL Robb's saline in a 25-cm<sup>2</sup> T-flask.
2. Irradiate with 24 kR X-rays or  $\gamma$ -rays; this is most easily done with a high-intensity cesium source.
3. Following irradiation, dilute the feeder cells in medium to a concentration of  $1-2 \times 10^6$  cells/mL.

#### 3.4.2.2. CLONING BY DILUTION

Cloning by dilution is a general procedure that will probably work for any *Drosophila* cell line. Cloning in soft agar is easier and less expensive, but it does not work for all cell lines (*see Note 21*).

1. Dilute the transformed cells in a feeder cell suspension, aiming for approximately one to two transformed cells per milliliter (*see Note 22*).
2. Plate the cell suspension into 96-well plates at 100  $\mu\text{L}$ /well. A multichannel pipettor is quite helpful for this procedure. Seal the plates with Parafilm, and incubate in a sealed plastic box at 25°C.
3. After 1–2 wk, examine the wells for growing clones. If the dilution is correct, then very few wells will have more than one clone, and these can easily be identified visually.
4. Once a clone occupies around 10% of the surface area of the well, it can be scaled up. We advise using the following steps: 0.3 mL/well in a 48-well plate; 0.5 mL/well in a 24-well plate; 1 mL/well in a 12-well plate; 2 mL/well in a 6-well plate; 5 mL in a 25-cm<sup>2</sup> T-flask, 10 mL in a 100-mm Petri plate.

#### 3.4.2.3. CLONING IN SOFT AGAR

The following procedure works well for S2 cells.

1. Prepare the feeder cell suspension in 1.25X medium.
2. Dilute the transformed cells in the feeder cell suspension, aiming for about 200 transformants per 8 mL (*see Note 22*).
3. On a 100-mm Petri plate, place 8 mL of the cell suspension in 1.25X medium on one side, and 2 mL of molten agar on the other. Gently and quickly mix the two pools of liquid, being careful not to continue mixing once the agar starts to gel.
4. Leave plates ajar in the sterile hood while the agar gels completely, and then store the plates in a sealed plastic food-storage box in a 25°C incubator for about 2 wk. The soft agar is very fragile, so it is essential to avoid jostling the plates.
5. After 2 wk, clones are small white spheres, visible in an inverted microscope, and visible to the naked eye with side-illumination. Once the clones reach about 1 mm in diameter, pick clones, using a Pasteur pipet or a glass capillary pipet, and transfer them to 100  $\mu\text{L}$  of medium in wells of a 96-well plate, pipetting up and down to try to break up the agar plug.
6. Scale up the clones exactly as previously described for cloning by dilution.

### 3.5. Induction

To induce expression from the Mt promoter, we normally add  $\text{CuSO}_4$  to a final concentration of 1 mM. In some cases expression may be improved by varying the concentration of  $\text{Cu}^{2+}$  or by substituting  $\text{Cd}^{2+}$ . The optimum time for inducing expression must be determined for each transgene that is expressed; it will depend on the half-life of the RNA and protein being expressed, and on the toxicity of the protein (*see Note 23*).

### 3.6. Miscellaneous Methods

#### 3.6.1. Robb's Saline (see Note 24)

1. Prepare 2 L part A: 6.08 g NaCl, 5.96 g KCl, 3.60 g glucose, 68.46 g sucrose, 0.59 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.49 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.29 g CaCl<sub>2</sub>·2H<sub>2</sub>O, H<sub>2</sub>O to 2 L.
2. Prepare 2 L part B: 0.57 g Na<sub>2</sub>HPO<sub>4</sub>, 0.10 g KH<sub>2</sub>PO<sub>4</sub>, adjusted to pH 6.75 with 1 M HCl, H<sub>2</sub>O to 2 L.
3. Autoclave part A and part B in separate 4-L Ehrlenmeyer flasks. Also autoclave an empty 4-L flask and sufficient bottles to store the saline.
4. Allow the two solutions to cool. Then mix them, and dispense into sterile bottles.

### 4. Notes

1. The only case in which plasmid copy numbers in transformed cells have been systematically measured is S2 cells transformed by calcium phosphate–DNA coprecipitation (2). In this situation, a cell typically incorporates approx 4000 copies of plasmid; if multiple plasmids are cotransfected, they are represented in approximately the same molar ratio as the input DNA. Thus, cells transformed with a 1:1 mixture of the expression plasmid and a selection plasmid will retain about 2000 copies of the expression plasmid, whereas a cell transformed with a 1:100 mixture of the same plasmids will end up with about four copies of the expression plasmid. The level of expression from the expression plasmid is roughly proportional to the number of copies. These predicted plasmid numbers are generally found as long as the expression plasmid does not prove harmful to the transformed cell; when that happens, either no transformants are recovered, or the transformants have greatly reduced levels of both plasmids.
2. Any insertion of exogenous DNA is mutagenic. With most transformation procedures, the transforming DNA forms one or a few long arrays whose insertion into the chromosome causes few mutations, and the number of mutations does not increase substantially with the amount of DNA incorporated. P elements, by contrast, are inserted individually, and transformation with a large number of P element copies generally causes lethality. Therefore cells transformed with P element insertions generally contain 1–10 copies. Within this very restricted range, copy number generally increases with increasing concentration of either the P element plasmid or the transposase plasmid.
3. The *Drosophila* Genomics Resources Center maintains a collection of over 100 *Drosophila* cell lines, including all those in common use; all are available for purchase, but a material transfer agreement is required in some cases. The *Drosophila* Genomics Resources Center also offers considerable user support, both in the form of protocols on its website (<http://dgrc.cgb.indiana.edu>) and as responses to individual queries. S2 and Kc are in the public domain and in widespread use; hence you may be able to obtain these lines from research labs that are using them. Other lines are less easily found from informal sources.
4. Hyclone has reported (59) that heat-inactivation is in general unnecessary and sometimes deleterious to growth of mammalian cell lines. Although we have not

tested untreated serum extensively, in our experience it is toxic to *Drosophila* cell cultures. We do not know the reason for the difference from the Hyclone observations, but it is possible that some toxic components of serum that are inactivated in cultures kept at 37°C, as suggested by Hyclone, remain active in *Drosophila* cell cultures which are maintained at 25°C.

5. Most suppliers of serum are willing to provide a small sample of serum for testing before the purchase of a large lot; the authors recommend this procedure, although they have never found a lot of serum that does not support the growth of S2 or Kc cells.
6. The original plasmids made by Bunch et al. (46) provide a polylinker for insertion of an open reading frame between a Mt promoter and a polyadenylation signal; some of these plasmids are available from the DGRC, and all can be requested from Dr. Bunch. More complex versions available in the Invitrogen kits are designed to produce fusion proteins with epitope tags or secretion signals.
7. Plasmids for strong constitutive expression using the act5C promoter vary from pPac and RAct-HAdh, which simply contain a short polylinker flanked by promoter and polyadenylation regions, to plasmids that use the Gateway™ technology to construct coding sequences incorporating a variety of epitope and fluorescent tags. Most of these plasmids are available from the DGRC; pAc5.1/V5-His is available from Invitrogen.
8. pHGCO and pHCO are the original plasmids used for methotrexate selection in *Drosophila* cell lines, expressing a methotrexate-resistant DHFR from a copia promoter (1); both are based on pBR322, and both have unusually low yields in plasmid preps. p8HCO (9) is simply the insert of pHCO subcloned into pUC8; plasmid prep yields of p8HCO are approx 10-fold higher than those for pHGCO and pHCO. At least five copies of the copia-DHFR expression sequence are required to confer methotrexate resistance in S2 cells (2). For transformation with a single copy of the plasmid, as in P element transformation, it is necessary to replace the copia promoter with a much stronger promoter, as in actDHFR (39).
9. Additional hygromycin selection vectors have been made that are designed for the insertion of the expression unit in the same plasmid (e.g., ref. 38), but these are not recommended because they lose the advantages of cotransfection for both ease of construction and flexibility in directing the copy number in the transformed cells.
10. The original protocol for transfection using calcium phosphate–DNA coprecipitation (60) used HEPES-buffered saline, which is retained in the Invitrogen kit; we have found BBS (61) to give transfection efficiencies about an order of magnitude higher than HEPES-buffered saline.
11. When MTX degrades, it becomes toxic even to MTX-resistant cells. Therefore, do not use MTX that has been stored at 4°C for more than 2 wk, and always protect MTX from light.
12. Forty-five to 50°C is the lowest temperature at which the agar is reliably liquid; at higher temperatures, the agar will cause excessive damage to the cells.
13. Although cells do not grow well on the surface of bacteriological grade Petri plates, there is no harm in using these plates for cloning in soft agar. Bacteriological grade plates are approx 10% of the cost of tissue culture-grade plates.

14. Cell lines can be stored indefinitely in liquid nitrogen or for a few months at  $-80^{\circ}\text{C}$ . We recommend that a few ampoules be frozen as soon as possible once a transformed line is established.
15. Cells can be thawed instead in a 60-mm Petri plate containing 4 mL of medium. We recommend using the T-flask because the latter can be sealed; this is particularly important for lines that are slow to recover from freezing and may have to remain in the original medium for 1 wk or more.
16. It is difficult to interpret measurements of transfection efficiency as fraction of cells expressing the trans-gene following transient transfection because the number of cells counted as “expressing” is greatly dependent upon the protein being expressed and the assay sensitivity. With the exception of specific cell lines that are recalcitrant to transfection by the calcium phosphate technique, 1–50% of cells can typically be detected as “expressing” the trans-gene using any of these techniques. When transfection efficiency is measured by enzymatic assay of the reporter in a cell extract, preliminary data suggests that the techniques all give roughly similar results, with the same caveat (Cherbas, L. and Osswald, M., unpublished observations).
17. As a first approximation, the amount of DNA taken up by the cells is determined by the concentration of DNA in the cell suspension; there is only a slight decrease in uptake per cell as the number of cells is increased. We have successfully performed electroporation with the cell concentration in the cuvet varying from about  $2 \times 10^6$  cells/mL to about  $2 \times 10^8$  cells/mL.
18. Søndergaard compared transfection efficiencies for several lipofection reagents on S2 cells (62). In one report, virtually all S2 cells transfected with Effectene expressed the trans-gene (*see* online supplement to **ref. 63**), but in our experience transfection efficiencies are lower with most cell lines. We have recently tested Cellfectin, FuGene 6, and Effectene (Qiagen) on about 20 cell lines of embryonic, imaginal disc, and central nervous system; preliminary results indicate that all of the reagents gave at least some transfection, with transfection efficiencies varying greatly with the combination of reagent and cell line, and that embryonic lines in general (including S2 and Kc) gave much higher transfection efficiencies than imaginal disc or central nervous system lines (Oswald, M. and Cherbas, L., unpublished observations).
19. Unfortunately, even clones of transformed cells are often not entirely homogeneous because of continuing rearrangements of the transformed array.
20. In general, we recommend retaining the selective agent during routine maintenance of transformed cells to guard against rare recombination events leading to loss of the transgenes. This is easy to do with an inexpensive reagent such as methotrexate, but it is prohibitively expensive with  $\alpha$ -amanitin. If you choose to grow transformed cells in the absence of the selective agent, then it is advisable to check the line occasionally to make sure that the transforming DNA has not been lost.
21. S2 cells can be cloned in soft agar; Kc cells cannot.
22. You can make a rough estimate about transformation efficiency by determining the frequency of expression of a reporter in a transient transfection, using the

same cell line and transfection technique. We advise cloning with using several dilutions in parallel to compensate for uncertainty in this estimate of transformation efficiency.

23. In stably transformed S2 cells, the nuclear receptor EcR, expressed from a Mt promoter, reaches its maximum level within 3 h (Mintzas, A. and Cherbas, L., unpublished observations). The membrane signaling protein Delta, expressed similarly, reaches its maximum level after about 12 h (Klueg, K., personal communication). In transient expression assays, luciferase expressed from a constitutive promoter continues to increase for at least 24 h (Cherbas, L., unpublished observations).
24. Robb's saline is a good all-purpose saline solution in which cells remain alive and healthy for several hours.

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## Transforming Lepidopteran Insect Cells for Improved Protein Processing

Robert L. Harrison and Donald L. Jarvis

### Summary

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. Methods for engineering these 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 and trafficking are described.

**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 baculovirus expression vector system (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 of the co- and posttranslational 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 specific protein processing capabilities of higher eukaryotes. For example, baculovirus-infected Sf-9 cells generally fail to  $\alpha$ -amidate recombinant proteins (2–4). Such limitations do not always reflect the general absence of a specific protein-processing pathway in lepidopteran insect cells, however. In fact, this is true of  $\alpha$ -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 (16).

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 (1,17–20).

One way to address the types of protein processing problems previously described 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 15. The resulting cell lines are designed to serve as improved hosts for the BEVS that have the ability to produce more accurately 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 (1) constructing constitutive expression plasmids encoding the desired protein

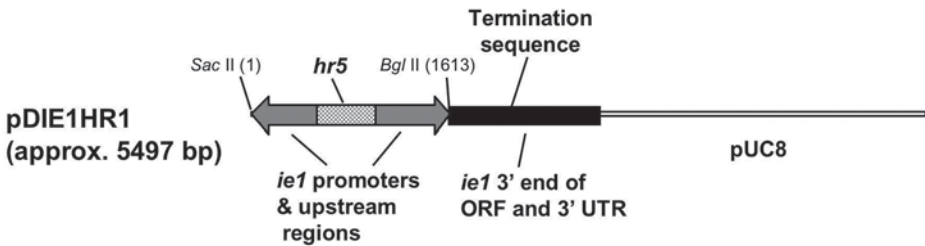


Fig. 1. Features of pDIE1HR1. This plasmid contains an *hr5* enhancer and two back-to-back *ie-1* promoters, as indicated. It includes a transcriptional termination signal downstream of the right-hand *ie-1* promoter, but not downstream of the left-hand promoter. *Sac*II and *Bgl*II sites are available for inserting genes of interest. The *ie-1* translational initiation codons downstream of both *ie1* promoters have been deleted in this construct.

processing functions, (2) using the resulting constructs to produce stably transformed lepidopteran insect cell lines, and (3) assessing the relevant properties of the new cell lines with respect to their application as improved hosts for the BEVS.

## 2. Materials

1. The cells, media, selection antibiotics, and other reagents used to isolate stably transformed insect cell lines are the same as those described in Chapter 15. In particular, the procedures described in this chapter require Hink's TNM-FH medium supplemented with 10% fetal bovine serum, 0.1% Pluronic® F68, 1.25  $\mu$ g/mL amphotericin B, and 25  $\mu$ g/mL gentamycin (referred to as complete TNM-FH). 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](http://www.fishersci.com)).
2. For viral plaque assays, we use SeaPlaque low melting point agarose (cat. no. 50101) from Cambrex Corporation (East Rutherford, NJ, [www.cambrex.com](http://www.cambrex.com)). 2X TNM-FH can be prepared as previously described (21) or purchased from a commercial vendor such as Sigma-Aldrich (St. Louis, MO, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)).
3. Plasmids: the pIEHR series of expression vectors (Chapter 15, Fig. 2), the dual gene expression vector pDIE1HR1 (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-ie1* enhancer-promoter arrangement (the pIEx series) is available for purchase from Novagen/EMD Biosciences (Madison, WI, [www.emdbiosciences.com](http://www.emdbiosciences.com)). In addition, expression plasmids (the pIB and pIZ series) that carry drug resistance genes for blasticidin S and Zeocin™ are available from Invitrogen (Carlsbad, CA, [www.invitrogen.com](http://www.invitrogen.com)).

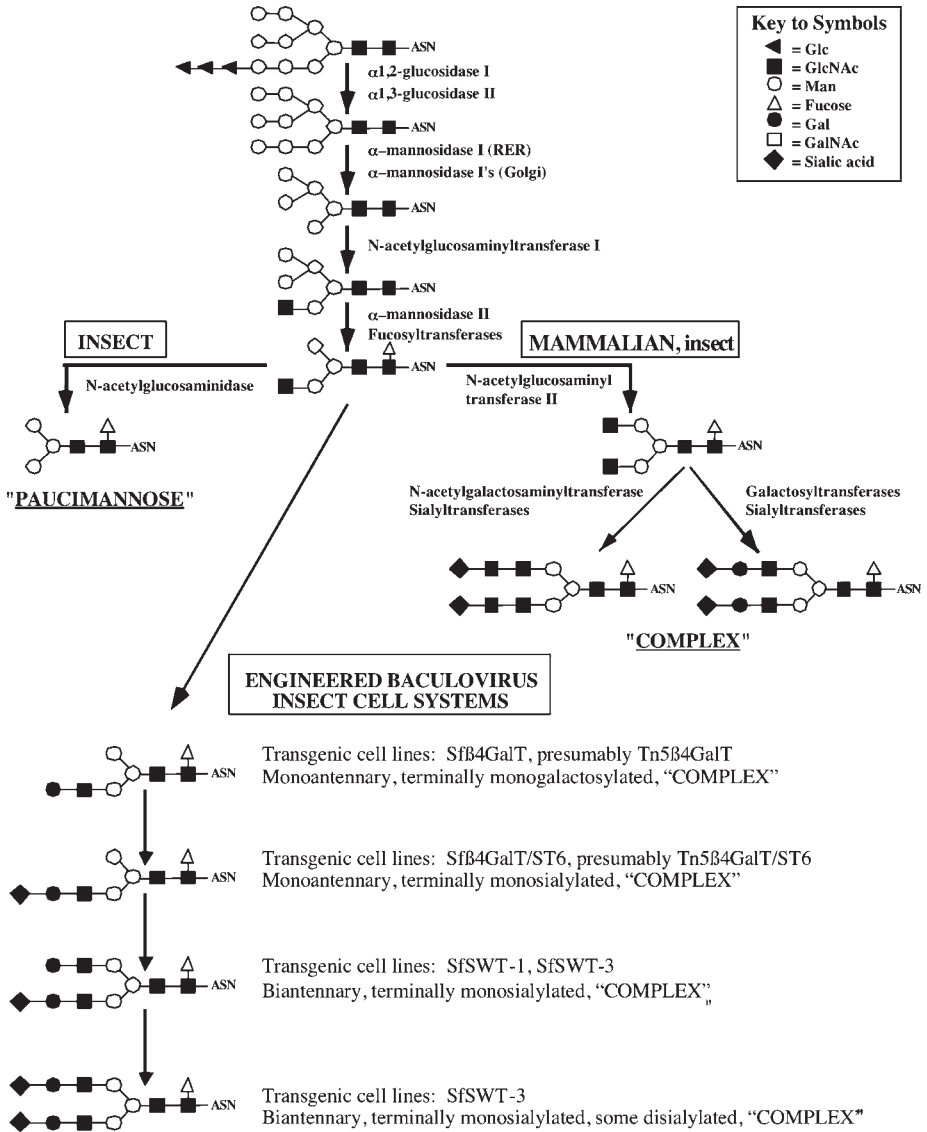


Fig. 2. *N*-glycosylation pathway in insects and mammals, and the *N*-glycans formed in transformed cell lines. (Reprinted from ref. 27 with permission from Elsevier.)

### 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 15. 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 an expression plasmid designated pDIE1HR1 (22). 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) cells (22,23).

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 sequentially, in a 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. This sequential approach also can be used to obtain 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, which include G418, hygromycin B, Zeocin, and blasticidin S, each have different mechanisms 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 (23–25).

#### 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, or RNase protection assays, as described in Chapter 15. In addition, because these transformed subclones are to be used as improved hosts for baculovirus-mediated recombinant protein production, other properties related to their ability to serve this host function must be examined.

### **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, or maximal achievable density. One also can imagine other phenotypic changes, such as differences in cell size, morphology, 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* Chapter 1 and **Note 3**).

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, as well as alternative methods for obtaining cell densities.
5. A transformed cell line population doubling time 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, 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 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 approx  $1.25 \times 10^6$  cells/mL.
3. Pellet the cells from each culture by low-speed centrifugation (500g for 3 min).
4. Resuspend the cell pellets in a small volume of complete TNM-FH. Infect at a multiplicity of infection of five by adding the appropriate volume of wild-type *Autographa californica* multiple nucleopolyhedrovirus. The final cell density after the addition of inoculum should be approx  $10^7$  cells/mL. Incubate each culture on a rocking platform for 1 h at 28°C.
5. Repellet 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* Chapters 4, 5, 10, 11, and 21 for various methods of titering virus).
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 may 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% sodium dodecyl sulfate.
9. Pellet the polyhedra by centrifugation at 5000g for 5 min.
10. Resuspend the polyhedra in 1 mL 0.5 M NaCl. Repellet the polyhedra.

11. Resuspend the polyhedra in 0.5 mL dH<sub>2</sub>O 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 in **Subheading 3.4**. Harvest aliquots of the infected cell cultures daily from 1–5 d postinfection, 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 clearly contains and expresses a new protein processing gene, but fails to process recombinant proteins encoded by the BEVS in the expected fashion. This could result from the inability of a protein-processing enzyme to accumulate in

the appropriate subcellular compartment or from the absence of various cofactors required for the enzyme to perform its function *in vivo*.

For these reasons, the ability of a new insect cell line engineered to have a new protein processing function to actually perform that function should be assessed using a model protein. The methods used for this experiment will obviously depend upon the function being assessed.

### **3.7. Examples of Stable Transformation for Improved Protein Processing**

#### *3.7.1. Sf-9 Cells Transformed for Altered Protein N-Glycosylation*

Because of 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). 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 general 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 (28). 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 (29).

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 (30). This results in the production of a common intermediate in both cell types. In mammalian cells, this intermediate is typically elongated by a series of glycosyltransferases to produce “hybrid” or “complex” *N*-glycans, which often contain terminal sialic acid residues that contribute a negative charge. In contrast, glycoproteins produced by established lepidopteran insect cell lines typically contain “high mannose” or “paucimannose” *N*-glycans (Fig. 2), with no terminal sialic acids. It remains to be seen whether or not insects, in general, have the ability to produce complex, terminally sialylated *N*-glycans like those found on mammalian glycoproteins (19). Although there is growing evidence that at least some insects and insect cell lines have some of the requisite machinery (31,32) and a limited capacity to produce sialylated glycoproteins (33–35), it is clear that the major processed *N*-glycan species found on most recombinant glycoproteins produced by baculovirus-infected insects and insect cell lines has the paucimannose, rather than the complex, terminally sialylated structure.

The first insect cell line to be genetically transformed for the purpose of extending its *N*-glycan processing capabilities was Sf $\beta$ 4GalT (36). This cell line consisted of Sf-9 cells transformed with an immediate early expression plasmid (37) encoding bovine  $\beta$ 1,4-galactosyltransferase under the transcriptional control of the *A. californica* multiple nucleopolyhedrovirus *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,  $\beta$ -linked galactose residues when it was produced during baculovirus infection of Sf $\beta$ 4GalT, but not Sf-9 cells. These results showed that the endogenous protein *N*-glycosylation pathway of Sf-9 cells could be extended by the introduction of a constitutively expressible mammalian glycosyltransferase gene.

Subsequently, Sf $\beta$ 4GalT cells were super-transformed with an immediate early expression plasmid encoding a rat  $\alpha$ 2,6-sialyltransferase (ST6GalI) to produce a cell line designated Sf $\beta$ 4GalT/ST6 (24). This new cell line produced terminally sialylated versions of gp64 and an additional model glycoprotein, GST-SfManI. The Tn-5 cell line was also successfully transformed with these same mammalian  $\beta$ 1,4-galactosyltransferase and  $\alpha$ 2,6-sialyltransferase genes using the dual immediate early expression plasmid previously described (22). The resulting cell lines also acquired the ability to produce terminally galactosylated and sialylated glycoproteins.

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 high-performance liquid chromatography and mass spectroscopy for this purpose and the results of these additional analyses revealed that the *N*-glycans synthesized by Sf $\beta$ 4GalT and Sf $\beta$ 4GalT/ST6 cells had, indeed, acquired terminal galactose and sialic acid residues, respectively, but only on one branch (25,38). Although the lower ( $\alpha$ 3) branch of the *N*-glycans produced by these cells was terminally galactosylated or sialylated, the upper ( $\alpha$ 6) 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 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 Sf $\beta$ 4GalT cells with immediate early expression plasmids encoding a comprehensive selection of mammalian glycosyltransferase

genes, including *N*-acetylglucosaminyltransferase I, *N*-acetylglucosaminyltransferase II,  $\beta$ 1,4-galactosyltransferase,  $\alpha$ 2,6-sialyltransferase (ST6GalI), and  $\alpha$ 2,3-sialyltransferase (ST3GalIV). The resulting cell line, designated SfSWT-1, produced complex, biantennary *N*-glycans in which the  $\alpha$ 3 branch terminated with sialic acid and the  $\alpha$ 6 branch terminated with galactose (25).

Interestingly, we found that Sf $\beta$ 4GalT/ST6 and SfSWT-1 cells produced terminally sialylated glycoproteins even though Sf-9 cells have no detectable CMP-sialic acid, which is 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, and CMP-sialic acid synthetase (23). 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  $\alpha$ 2,6-sialylated on the  $\alpha$ 3 branch. SfSWT-3 cells produce an additional, 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  $\alpha$ 2,3-sialyltransferase activity by the immediate early expression plasmid encoding ST3GalIV (Harrison and Jarvis, unpublished data), suggest that this plasmid cannot induce this activity in Sf-9 cells. Thus, we have initiated further engineering efforts in an attempt to introduce a functional  $\alpha$ 2,3-sialyltransferase gene into Sf-9 cells and obtain a cell line that can produce recombinant glycoproteins with disialylated, biantennary *N*-glycans.

All of the transformed cell lines described above were able to support baculovirus infection at similar levels. This was somewhat surprising, as Sf $\beta$ 4GalT/ST6, SfSWT-1, and SfSWT-3 cells should terminally sialylate endogenous membrane glycoproteins, which would dramatically alter the surfaces of these cells. This might have been expected to inhibit virion attachment, penetration, assembly, or release, as previously discussed. It also was noted that SfSWT-1 cells were noticeably smaller than Sf-9 and SfSWT-3 cells and achieved higher final densities than Sf-9 and SfSWT-3 cell lines after an initial period of slower growth (23). Finally, the expression levels obtained for one model glycoprotein was found to be similar after infection of Sf-9, SfSWT-1, and SfSWT-3 cells with the BEVS, although there were some differences in expression kinetics (23).

### 3.7.2. Sf-9 Cells Transformed for Improved Protein Folding

It has been suggested that deficiencies in protein folding in baculovirus-infected insect cells might account, at least in part, for the general inability to produce large quantities of functional secretory pathway products in this system (16). In an attempt to address this problem, Lenhard and Reiländer (44) transformed Sf-9 cells with a gene encoding NinaA of *Drosophila melanogaster*. NinaA is a type I membrane protein with homology to cyclophilins, which are proteins that have peptidyl-prolyl *cis-trans* isomerase activity and a number of functions, including protein folding (45). NinaA has been shown to be required for the accumulation and trafficking of the *D. melanogaster* rhodopsin, Rh1 (46).

The resulting transformed Sf-9 derivative, designated Sfn, expressed NinaA under the transcriptional control of the *ie-1* promoter at levels that were detectable by immunoblotting analysis (44). Sfn cells grew more slowly than Sf-9 cells, accumulating to half the concentration 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 the BEVS. However, there was a larger (5-fold) increase in dopamine uptake by Sfn cells infected with the BEVS expressing the human dopamine transporter, as compared to Sf-9 cells infected with the same virus. The authors concluded that the Sfn cells produced more functional, plasma membrane-localized dopamine transporter owing to the coexpression of NinaA activity by these cells.

It remains unclear whether the increase in functional dopamine transporter observed in Sfn cells resulted specifically from improved protein folding. Dopamine uptake by the infected Sfn cells was reduced by 40% after treatment with cyclosporin A, an inhibitor of cyclophilin peptidyl-prolyl *cis-trans* isomerase. In contrast, cyclosporin A had no effect on dopamine uptake by infected Sf-9 cells. The observation that cyclosporin A inhibition of dopamine uptake occurred only with Sfn cells suggested that a cyclophilin-specific isomerase activity was responsible for the increased uptake observed with Sfn. However, it has been shown that NinaA is quantitatively required for the trafficking of rhodopsin Rh1, indicating that it acts as a chaperone, rather than a folding enzyme for Rh1 (47). Thus, it is possible that NinaA was functioning in this same fashion to enhance the activity of the baculovirus-expressed dopamine transporter in Sfn cells.

## 4. Notes

1. Generally, our experience with transfecting cells with multiple plasmids suggests that (1) it is necessary to screen more clones to identify one that has incorporated every plasmid, and (2) 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 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 approx 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  $0.3\text{--}0.5 \times 10^6$  cells/mL and these cells typically achieve a density of  $2\text{--}4 \times 10^6$  cells after 2 d of growth in 50-mL shake-flask cultures. However, we have found that some stably transformed cell lines, such as SfSWT-3 (23), benefit from a slightly higher seeding density 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 endo- or exoglycosidases that will remove all or part of the *N*-glycan prior to the lectin-binding assay.

## Acknowledgments

DLJ gratefully acknowledges the National Institutes of Health (GM49734), the National Science Foundation (BES-9814157 and BES-9818001), and the United States Department of Agriculture-National Research Initiative (89-37266-4935 and 95-37302-1921/2658) for supporting work in his lab. 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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## Introduction to the Use of Baculoviruses as Biological Insecticides

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### Summary

Baculoviruses are widely used both as protein expression vectors and as insect pest control agents. This section provides an overview of the baculovirus lifecycle 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. Formulation and application of baculoviruses for pest control purposes are described elsewhere (1).

**Key Words:** Baculovirus insecticides; recombinant baculovirus; insect pest management.

### 1. Introduction

Baculoviruses have been isolated from more than 400 insect species, mainly within the Lepidoptera (butterflies and moths) (2,3). The two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), within the family *Baculoviridae* are identified by occlusion body (OB) morphology with single (GV) and multiple (NPV) virions (occlusion-derived virus, ODV) occluded in granules and polyhedra, respectively. Baculoviruses have been used extensively for control of insect pests in a diverse range of habitats, including forests and greenhouses. 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 pine forests in Germany (4). Both NPVs and GVs have been registered for use as microbial pesticides. In 1975, the first virus insecticide, Elcar™, was registered in the United States for use against the cotton

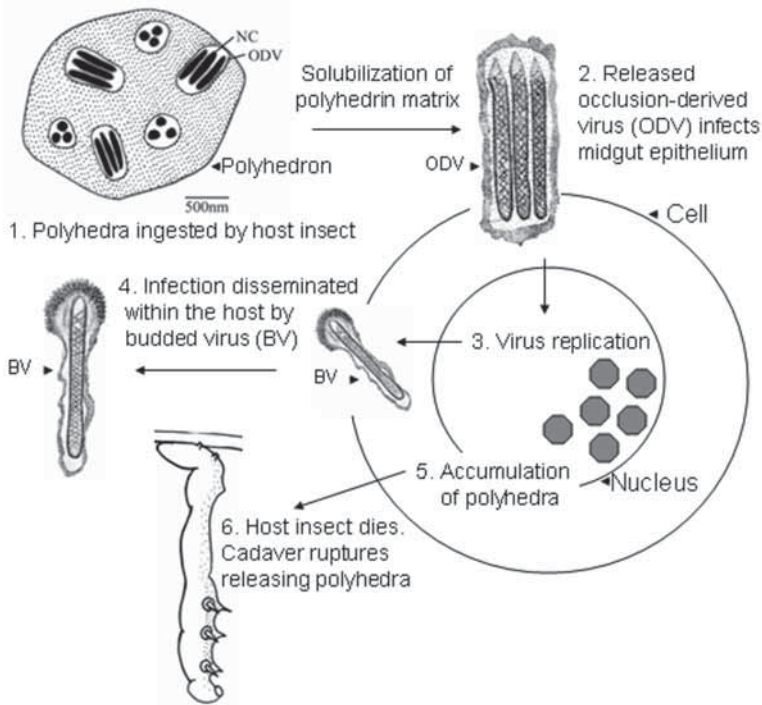


Fig. 1. Life cycle of a multiple nucleopolyhedrovirus (MNPV). MNPV have multiple nucleocapsids (NC) within occlusion-derived virus (ODV). The two baculovirus phenotypes, ODV and budded virus (BV), are illustrated showing the NC within each virion type.

bollworm *Helicoverpa zea*. One of the most successful examples of use of a wild-type baculovirus is application of *Anticarsia gemmatalis* multiple NPV for management of the velvet bean caterpillar in soybean crops in Brazil, where it is applied to more than 1 million hectares annually (5). A second example of successful use of a baculovirus insecticide is on cotton in China against *Helicoverpa armigera* (6).

## 2. Baculovirus Life Cycle

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 ODV are released (Fig. 1). The ODV pass through the peritrophic membrane and enter the columnar cells of the midgut epithelium (7–9). Virus replication takes place in the nucleus.

Initial rounds of replication result in production of a second viral phenotype, budded virus (BV) (**Fig. 1**). BVs circulate in the hemolymph and initiate sites of secondary infection throughout the host body. The midgut, hemocytes, tracheal matrix, and fat body cells are the most readily infected in susceptible hosts (**7**).

A switch 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 at very late stages of infection and polyhedra are released into the hemolymph at advanced stages of disease. Larvae appear swollen and become lethargic, and typically exhibit negative geotropism prior to death, which may facilitate polyhedra dispersal. The cuticle of the cadaver ruptures easily thereby releasing polyhedra into the environment. The time taken for the virus to kill the host may take anywhere from 5 d to 3 wk according to the virus–host combination and environmental conditions. This time allows for extensive virus replication while the host continues to feed, with a single cadaver yielding as many as  $10^{10}$  polyhedra (**10**).

### 3. Pros and Cons for Use of Baculovirus Insecticides

Advantages of using baculovirus insecticides include their specificity and safety, i.e., they are harmless to nontarget organisms such as vertebrates and plants (**11**). They persist for many years in environments such as the soil where they are protected from light (**12**). In addition, baculoviruses are ideal for use in integrated pest management because they can be used in conjunction with chemical insecticides, or other biological control agents (**13**). Baculoviruses are not currently in widespread use, however (**5,14**). Limitations to their use include problems with long term storage, high cost of production relative to classical chemical pesticides, persistence in the field when exposed to ultraviolet light, and the relatively slow speed of kill of the targeted pest (**15**). The narrow host range is restrictive for management of multiple pest species in a cropping system, and farmer expectations are based on the use of fast-acting classical chemical insecticides. Commercial scale production and formulation of baculovirus insecticides have also met with technical challenges, some of which have now been addressed.

Baculoviruses have a slow speed of action compared to the classical synthetic chemical insecticides. Infected larvae continue to feed for several days until their death and may cause significant damage. 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.

#### 4. Genetic Enhancement of Baculovirus Insecticides

More than 30 recombinant baculoviruses have been constructed with the specific goal of enhancing the insecticidal properties of the virus (16). 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 (17), and improvement of virus stability (18,19) 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, prothoracicotropic hormone, decreased the pathogenicity of the baculovirus (20); a baculovirus expressing a maize protein that disrupts the inner mitochondrial membrane was insecticidal (21); a catalytically inactive insect enzyme, juvenile hormone esterase was toxic to baculovirus infected larvae (22). In the event that a baculovirus-expressed protein exhibits toxicity to insect cells in culture, testing the recombinant baculovirus for insecticidal effects using the protocols described in Chapter 20 may be warranted.

The methods developed for constructing recombinant baculoviruses for protein expression purposes (23–25) have been used for production of recombinant baculovirus insecticides. The main difference in the approach toward the two goals is the requirement for baculovirus insecticides to express the polyhedrin gene. The polyhedrin matrix that embeds the ODV confers protection against desiccation and freezing (26). Polyhedrin is not needed for *in vitro* infection of insect cells. For recombinant protein expression purposes the foreign gene is commonly inserted into the baculovirus genome in place of the polyhedrin gene, for protein expression driven by 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; (3) incorporation of a toxin into the OB. Only a few key examples will be described here. For comprehensive information on other recombinant baculovirus insecticides, the reader is referred to Kamita et al. (16).

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 (16,27).

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 (28,29), LqhIT2 derived from a scorpion (30,31),  $\mu$ -Aga-IV derived from a spider (32), and As II and Sh I derived from sea anemones (32). 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 (27,33). 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 (16). 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; neurotoxins that paralyze larvae for example may show a greater reduction in feeding damage than expected from their speed of kill (34).

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 (35,36). 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.

Another effective strategy for increasing the speed of kill has been incorporation of a Bt toxin into the polyhedrin matrix. Chang et al. (37) 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 mid-gut epithelial cells and cause osmotic cell lysis. The presence of the Bt toxin in the polyhedrin matrix reduced the  $LC_{50}$  of the baculovirus approx 100-fold, and the speed of kill was improved 63% compared to the wild-type virus (37).

Recombinant baculovirus insecticides have been tested in the field in the United States and in the United Kingdom. None, however, have been registered for commercial use in these countries. Trials in the United States demonstrated that recombinant baculoviruses are competitive with fast-acting chemical insecticides under field conditions in terms of preventing economic loss (38,39). Trials of recombinant baculovirus insecticides are ongoing in

China (6,40) and practical use on a wide scale may be seen in this country. 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.

### Acknowledgments

This work was supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Agreement no. 00-39210-9772 as well as Hatch Act and State of Iowa funds.

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## Baculovirus Insecticide Production in Insect Larvae

Nikolai van Beek and David C. Davis

### Summary

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 vivo* poses specific problems, because of the short survival time of the production host.

In this chapter, benchtop-scale production of two typical baculoviruses is described. First, the authors describe the production of wild type *Helicoverpa zea* nucleopolyhedrovirus in larvae of the bollworm, *H. zea*. Larvae of this species are very aggressive and need to be reared in isolation from each other. The authors then describe the production of a recombinant *Autographa californica* multiple nucleopolyhedrovirus in the noncannibalistic cabbage looper, *Trichoplusia ni*. The recombinant baculovirus encodes the insect-specific scorpion toxin LqhIT2, which severely limits progeny virus production. The tetracycline transactivator system enables the production of wild-type quantity and quality product while toxin expression is repressed.

**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), United States (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 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, 100 tons of bioinsecticide based on the *Helicoverpa armigera* nucleopolyhedrovirus (NPV) 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, approx 2 million hectares of soybeans are treated with product manufactured by a number of companies, one of which (Coodetec) has the capacity to manufacture 1.4 million hectare treatments per year. The product is also sold in neighboring countries (i.e., Paraguay and Bolivia). In India, there are several companies involved in baculovirus production, mostly *H. armigera* NPV. 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 United States, 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 (2). 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.

Because 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 18). Eventually, recombinant viruses expressing scorpion and spider toxins showed such promise that agrochemical companies initiated research and development 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 (3). Although American Cyanamid focused on *in vitro* 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 approx 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 (4). 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 quinquestriates hebraeus* T2, time-to-kill is reduced by 70% (5) 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.

The author 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](http://www.benzonresearch.com)) and Bio-Serv (Frenchtown, NJ, [www.bio-serv.com](http://www.bio-serv.com)).
2. Insect rearing trays: approximate dimensions of the wells in the trays, 1" × 1" × 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, cat. no. 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, cat. nos. 9050 and 9146, respectively).
6. Transfer forceps (e.g., BioQuip Products, Rancho Dominguez, CA, [www.bioquip.com](http://www.bioquip.com); cat. no. 4750).
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 cover slips: e.g., Bright Line Counting Chamber (Hausser Scientific, Horsham, PA; cat. no. 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](http://www.miami-aquaculture.com), Boynton Beach, FL) or four layers of cheesecloth (e.g. [www.thegadgetsource.com](http://www.thegadgetsource.com), Ashville, NC).

### 2.4. Production of Recombinant AcMNPV in *T. ni* Larvae

1. Doxycycline: 5 mg doxycycline (Sigma Aldrich) per milliliter 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 (6,7), develops from egg to pupa in about 16 d. The insect spends approx 2 d in the egg stage, followed by 12 d in the larval stage as the larva



Fig. 1. Early fifth instar *Helicoverpa 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.

progresses through five larval instars (Fig. 1). At the end of the fifth instar the insect enters a prepupal stage for approx 2 d. Metamorphosis takes place in the pupal stage, and after about 8 d the adult insect emerges. Unlike *T. ni*, *H. zea* larvae are extremely cannibalistic (8), 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.

### 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 next (see Note 2).

1. Determine the quantity of *H. zea* eggs. Ten eggs weigh approx 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 15 g grits and mix until clumps have disappeared.
4. Mix *H. zea* eggs and moistened grits (3.4 mg eggs per gram) and incubate for 18–24 h at 29°C and 60% relative humidity (RH).
5. If prepared insect diet is purchased ready for use in multicellular trays (Bio-Serv), proceed to **step 9**. The diet may also be prepared from a dry bulk premix 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 (Chapter 13, **Subheading 3.1.1**).
6. After preparation, place the liquid diet in a water bath at approx 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 preincubated 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 d 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  $\mu$ L stock suspension to 9.9 mL water. Mix thoroughly and then prepare five serial 10-fold dilutions (1 mL virus suspension in 9 mL water). Mix well before each subsequent dilution.
3. Before use, clean the hemacytometer and the cover slip with 70% alcohol and blot dry with tissue. Place the cover slip 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  $\mu\text{L}$  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  $\times 400$  magnification (*see Note 5*).
5. After vortexing the highest dilution of one series, withdraw 10  $\mu\text{L}$  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 cover slip, 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 less than 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 counted is  $2 \times 10^{-5}$  mL (a grid of  $0.1 \times 0.1$  cm, and a chamber depth of 0.01 cm, with 5 of the 25 squares of the grid counted).

### 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  $\mu\text{L}$  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 cheese-cloth) 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 approx 400 mL of the homogenate into 500-mL centrifuge bottles. Centrifuge at 12,000g 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 vol of 50% glycerol before storage at –20°C [*see Note 8*].)
8. The OB concentration in the paste may be determined by collecting three subsamples from the homogenized paste and counting the number of OBs present. If the paste subsamples cannot be quantified immediately, then they may be stored for up to 7 d 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 (9)—is the main cause of decreased yields in larvae infected with recombinant virus. The reduction in yield may be up to 90%, 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 LqhIT<sub>2</sub> (10,11), these effects are presumably owing 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<sub>2</sub> (7) under control of the tetracycline transactivator (12). This method has not been published, but it has been described in a patent (13). The following protocol should yield from  $5 \times 10^{11}$ – $10^{12}$  AcNPV-LqhIT<sub>2</sub> 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 in **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  $\mu$ L 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 next (see **Note 3**).
6. Incubate the cups at 28°C, 30–40% RH for approx 120 h, and begin monitoring for death at 96 h after inoculation.

### 3.7. Larval Harvesting and Downstream Processing of AcNPV-LqhIT<sub>2</sub> OBs

1. Larval harvesting and downstream processing are done as described in **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.

**Table 1**  
**Weight and Head Capsule Width for Each Instar of *Helicoverpa zea***  
**and *Trichoplusia ni* Larvae**

Stage (instar)	<i>H. zea</i>					<i>T. ni</i>				
	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th
Head capsule width ( $\mu\text{m}$ )	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

- It is possible to simplify this method, especially when the eggs arrive while still attached to the substrate. Although *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 3.1.2**. However, because of 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**.
- 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* (**14**) and *T. ni* larvae (**15**).
- H<sub>z</sub>NPV OBs are uniform in size and roughly 1  $\mu\text{m}$  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 particles in the range of 1 to 5  $\mu\text{m}$ . For the larger OBs, one may be able to see under the microscope their irregular, polyhedral shape.
- 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  $9 \text{ mm}^2$ . 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, whereas 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 ( $200 \mu\text{L}$ ) at half the concentration ( $10^4$  OBs/mL), 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  $-20^\circ\text{C}$ . 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).

## Acknowledgments

We thank Dr. Ian Smith (Nara Institute of Science and Technology, Japan) for reviewing the manuscript. We thank Drs. Andy Cherry, Michael Dimock, David Grzywacz, Flavio Moscardi, Madoka Nakai, Thi Tu Cuc Nguyen, Philip Sridhar, Xulian Sun, and Kees van Frankenhuyzen for providing information about baculovirus production and commercialization in various countries in the world.

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## Evaluation of the Insecticidal Efficacy of Wild-Type and Recombinant Baculoviruses

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### Summary

A considerable amount of work has been done during the last 20 yr 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 time of larvae infected at a fixed dose ( $ST_{50}$ ), and feeding damage incurred by infected larvae. In this chapter, protocols are described for a variety of bioassays and corresponding data analyses for assessment of the insecticidal activity or host range of baculovirus insecticides. Methods are also provided for baculovirus inoculation of larvae using a microapplicator for determining  $ST_{50}$  or for examining physiological effects.

**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, including insertion of genes encoding insect specific neurotoxins, hormones, or enzymes. Of the approx 30 optimized 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 lethal time ( $LT_{50}$ , where test

**Table 1**  
**Recommended Methods for Determination of Lethal Dose or Survival Time Parameters for *Heliothis virescens*<sup>a</sup>**

	Lethal dose/lethal concentration	Survival time	Subheading in chapter
Droplet feeding	L1–L3 <sup>b,c</sup>	L1–L3	<b>3.2.</b>
Diet cube	L3–L5	Not applicable	<b>3.3.</b>
Microapplicator	Not applicable	L4–L5 <sup>d</sup>	<b>3.4.</b>

<sup>a</sup>Procedures may vary for other species according to the size of test larvae and number of instars.

<sup>b</sup>LC data generated by droplet feeding methods can be converted to lethal dose data if the volume ingested is known (see **Subheading 3.2.**).

<sup>c</sup>L: Larval instar; n: number of instar. For example, L2, second instar.

<sup>d</sup>Injection of budded virus or oral inoculation of polyhedra (preferred method).

insects are continually exposed to virus) or median survival time (ST<sub>50</sub>, where larvae are infected at the beginning of the bioassay), 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, 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 via droplet feeding (6), infection of larvae via ingestion of a virus-treated plug of diet, and for assessment of feeding damage to leaves (2). Methods recommended for use with different larval instars are shown in **Table 1**. Methods are also described for calculation of the toxicological parameters LC<sub>50</sub>, LD<sub>50</sub> and ST<sub>50</sub> using appropriate statistical programs such as POLO-PC (7–9), SAS (10), and the S-Plus Kaplan-Meier Program (11).

## 2. Materials

1. Baculoviruses: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), wild type clone C6, and the recombinant baculovirus AcMLF9. ScathL that expresses a cathepsin-L-like, basement membrane-degrading protease (ScathL) (2).
2. Insects: *Heliothis virescens* (tobacco budworm). Eggs can be purchased from suppliers such as Bio-serv (Frenchtown, NJ) and Agripest (Zebulon, NC).
3. Insect rearing supplies: incubator; artificial diet for *H. virescens* is prepared using premixed diet powder (Bio-serv) according to the manufacturer's instructions; heavy duty blender (1 and 4 L); clear plastic Rubbermaid trays with lids; 1-oz plastic cups (with lids); plastic cup tray (30 cells) (Bio-serv); knife; brushes.

4. Supplies for preparation of polyhedra (occlusion bodies): 15- and 50-mL polypropylene or glass tubes; a dounce homogenizer with a pestle; an overhead stirrer; 10% sodium azide stock solution stored at 4°C; 0.1 and 0.5% SDS; 0.5 M NaCl; hemocytometers; inverted microscope (Nikon, TMS, Japan).
5. Bioassay supplies: food coloring dye (blue); 60-mm Petri dish; automatic microdispensing system PAX 100-2 (microapplicator, Burkard Scientific, Uxbridge, Middlesex, UK); plastic tuberculin syringes (1 cc) with 28.5-gauge needles (sharp tip); plastic tuberculin syringes (1 cc) with 32-gauge needles (blunt-tip, Popper & Sons, Inc., New Hyde Park, NY); LI-COR 3100 area meter (LI-COR Inc., Lincoln, NE).
6. Statistical analysis supplies: POLO-PC probit analysis program (LeOra Software, Berkeley, CA) (8), SAS software (SAS Institute Inc.) (10), and S-plus Kaplan-Meier program (v6.0.1) (11).

### 3. Methods

There are several techniques that can be used for determining median lethal dose or median survival time. The technique used depends in part on the stage of the larvae to be tested; e.g., use of the microapplicator is recommended for larger, late instar larvae (Table 1).

#### 3.1. Preparation of Polyhedra for Bioassay

Although it is recommended that host insect-derived polyhedra (occlusion bodies) be used for bioassays (12), polyhedra derived from insect cell culture can also be used. Recombinant baculovirus insecticides generally carry the polyhedrin gene, although there are some exceptions (13,14). Quantification of the occlusion-derived virus (ODV) of baculoviruses lacking the polyhedrin gene is complex and will not be addressed here.

##### 3.1.1. Insect Rearing

1. *H. virescens* eggs are typically laid on cloth. Sterilize a clear plastic Rubbermaid (or similar) tray and lid by rinsing in ethanol. When the tray is dry, transfer the egg sheet to the tray using gloves.
2. Cover the top of the tray with a sheet of plastic and put the lid on the tray over the plastic sheet. Place the tray in a growth chamber at 28°C with a 12:12 (L:D) light period. If the eggs are light green, then they may hatch in 2–3 d. To delay hatching, incubate at 15°C.
3. Before eggs hatch, cut the insect diet into cubes (approx 15 mm<sup>3</sup>) and distribute the diet cubes to 1-oz plastic cups or other containers for rearing of larvae. The cups are held in a 30-cell plastic tray.
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 clean beaker, and fill the beaker with about 10 mL of deionized dH<sub>2</sub>O), then rinse the brush

with ethanol followed by distilled water. Use the brush to transfer one larva to each diet cube. Wetting the brush on the moist diet facilitates the transfer of larvae.

5. For amplification of polyhedra, 30–60 larvae are generally needed per virus. It is recommended that you start with 60–120 larvae per virus. Some larvae may be lost as a result of handling death or escape. Seal the cups with lids and place the trays in the 28°C incubator, until the larvae reach fourth instar.
6. If you have not worked with *H. virescens* before, then inspect the larvae carefully twice each day. *H. virescens* larvae go through five developmental stages (instars) that are distinguished by the width of the head capsule. Although there is variation in the size of the body within an instar, the head capsule remains the same and only increases in size at the molt from one instar to the next. See **Note 1** for more information about how to distinguish the larval instars of *H. virescens*.
7. For polyhedra production, early fifth instar larvae can be infected either by injection of budded virus (BV) into the haemolymph or by oral infection with polyhedra.

### 3.1.2. Polyhedra Production

1. The virus inoculum used to infect larvae for production of polyhedra can be derived from insect cell culture, or from virus-killed larvae. To isolate polyhedra from cultured cell pellets, seed Sf-21 or other susceptible cells in a 75-cm<sup>2</sup> flask(s) at approx  $5 \times 10^6$  cells per flask.
2. Following attachment of the cells, infect the cells at approx 2 pfu/cell and place the flask(s) in the 28°C incubator.
3. When polyhedra are present in almost all cells, harvest the cells and place in a 50-mL polypropylene conical tube and store at 4°C for 2 d. 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 900g for 10 min at 4°C. Transfer the supernatant that contains budded virus 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°C. Alternatively, budded virus can be used for insect infection using microapplicator-assisted injection (see **Subheading 3.4.**).
6. 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 pre-molt stage, return them to the 28°C incubator and allow to molt overnight.
7. The following morning, use a scalpel to make small cubes of diet (approx 2–3 mm<sup>3</sup>) and place one in each cup with each larva.

8. Transfer the required number of polyhedra (500,000) on to the diet cube in a small volume (i.e., 1  $\mu$ L). The larvae will be hungry and will promptly consume the virus-contaminated cube of diet.
9. When all the larvae have consumed the virus-contaminated diet, provide excess diet, and return larvae to the 28°C incubator. If you miss the premolt stage, then proceed as described—the larvae should still be susceptible to a dose of 500,000 polyhedra, although susceptibility decreases as the larvae age.
10. The infected fifth instar larvae should die 6–8 d post-infection (d pi) for wild type AcMNPV C6 and 3–4 dpi 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 if the larvae have an odor.
11. 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.
12. Fix the pestle into the overhead stirrer, insert it into the homogenizer tube and then move the homogenizer tube up and down at least 10 times to grind the cadavers and release the polyhedra at a suitable spinning speed. Do not grind the tissues excessively otherwise contaminating fine debris will go through the cheesecloth filter with the polyhedra at the next step.
13. 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.
14. Filter the ground larvae through at least five layers of cheesecloth using a funnel.
15. 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.
16. 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 900g for 10 min at 4°C. Decant the supernatant into a bleach solution.
17. Wash the gray pellet of polyhedra using 0.5% SDS followed by 0.5 M NaCl solution, and repeat until the supernatant becomes clear as necessary.
18. Finally, wash once using sterile dH<sub>2</sub>O. Resuspend the final pellet in an appropriate volume of sterile dH<sub>2</sub>O supplemented with 0.02% sodium azide as preservative (see **Note 2**) and store at 4°C for later use.

### 3.2. Droplet Feeding Bioassay

The droplet feeding bioassay, which was developed by Hughes and Wood (6,15), can be used to determine either the median lethal concentration or median survival time of baculoviruses in either neonate or later instars (16). The advantage of this technique is that the virus inoculum is ingested over a short period of time (10–15 min), which is particularly important for determi-

nation of survival time responses. In this section, the procedures for a lethal-concentration bioassay are presented. For estimation of the median lethal dose using this technique, the amount of fluid ingested must be quantified (17). Alternatively, use of the diet plug inoculation technique for estimation of lethal dose is described in **Subheading 3.3**. The survival-time bioassay and data analysis are described in **Subheadings 3.4** and **3.6.3**. For use of the droplet feeding bioassay for  $ST_{50}$  analysis, larvae are infected at a fixed dose of  $LC_{99}$  and monitored regularly (every 4–8 h, with more frequent observations when the mortality rate is high).

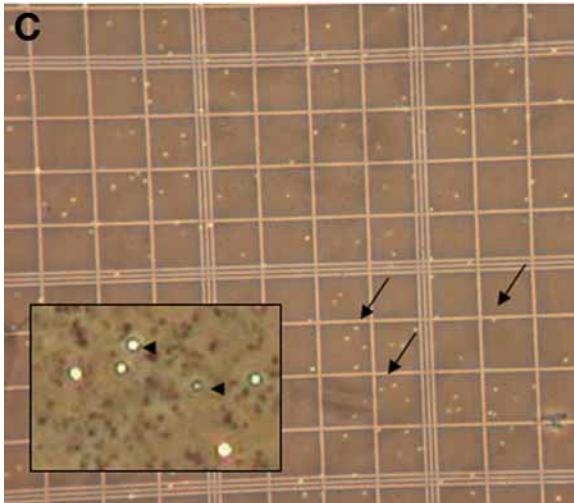
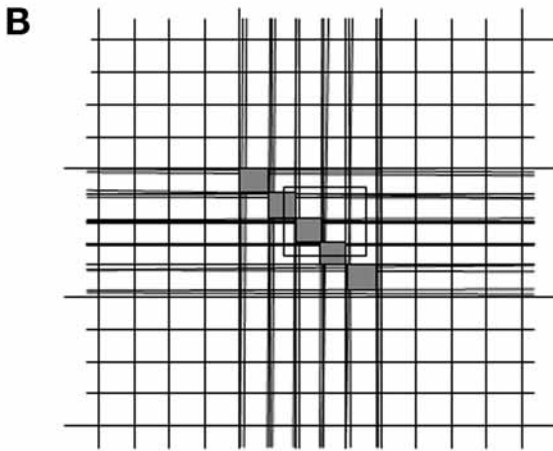
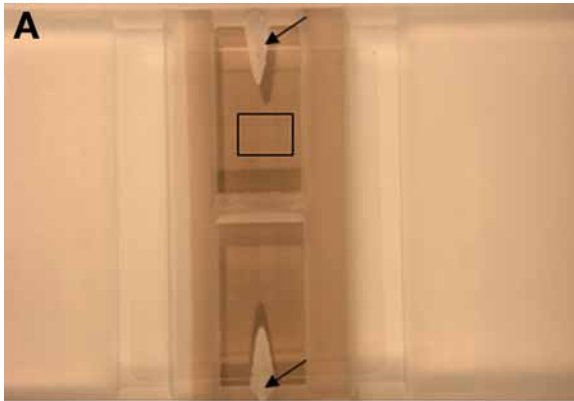
### 3.2.1. Quantification of Polyhedra and Determination of Concentration Range

1. Polyhedra are counted by using a hemacytometer and inverted microscope (**Fig. 1**). 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 pipet.
2. Examine the hemacytometer under the microscope with a  $\times 20$  objective on the inverted microscope. The polyhedra in the large gridded central square will be counted. Polyhedra appear as small, polyhedral objects in the field of view (**Fig. 1**).
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. 1B**). 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. The concentration in polyhedra per milliliter can be obtained using the following formula:  

$$Y = \text{mean polyhedra per small square} \times 25 \text{ (total number of squares)} \times \text{dilution factor} \times 10^4$$
, where  $Y$  is the concentration in polyhedra per milliliter.
4. Early instar larvae are generally used for droplet feeding bioassays (**Table 1**). After hatch, allow neonate larvae to starve overnight. If second instar larvae are to be used, then place neonate larvae in 48- or 24-well plates with diet. After 1 d at  $28^\circ C$ , remove the diet and starve the second instar larvae overnight.

---

Fig. 1. (*opposite page*) Quantification of polyhedra using a hemacytometer. (A) Hemacytometer. Place the cover slip 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 cover slip. Detail of the boxed area is shown in B. (B) Diagram of the grid that you will see 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).



5. The concentrations of AcMNPV polyhedra 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*, concentrations of  $0.5 \times 10^5$ ,  $2.0 \times 10^5$ ,  $5.0 \times 10^5$ ,  $10 \times 10^5$ , and  $20 \times 10^5$  polyhedra per milliliter would be appropriate. These concentrations are designated as  $M_i$  ( $i = 1, 2, 3, 4, 5$ , i.e., M1, M2, M3, M4, and M5).
6. To make the dilution, first determine the concentration ( $S$ , polyhedra/ $\mu\text{L}$ ) of stock polyhedra using a hemacytometer and microscope. Using the formula:  $V_i$  ( $\mu\text{L}$ ) =  $(400 \mu\text{L} \times M_i)/S$ , to calculate the volume ( $V_i$  in  $\mu\text{L}$ ) of stock polyhedra to make a 400  $\mu\text{L}$  diluted suspension with each desired concentration ( $M_i$ ). Add a  $V_i$  volume of stock polyhedra to a 1.5-mL centrifuge tube containing  $(400 - V_i)$  volume ( $\mu\text{L}$ ) of  $\text{dH}_2\text{O}$ , vortex for seconds to mix it, then add 16  $\mu\text{L}$  of food coloring dye (i.e., 4%, v/v, blue color). Four hundred microliters of  $\text{dH}_2\text{O}$  with dye is used for a negative control treatment.

### 3.2.2. Infection by Droplet Feeding and Data Collection

1. Make two concentric circles of small droplets (1–2  $\mu\text{L}$  per droplet) of virus suspension or negative control treatment in a 60-mm Petri dish (**Fig. 2**).
2. Transfer 15–20 neonates or second instars to the middle of the concentric circles (*see Note 3*). Place the lid on the dish and seal with Parafilm to prevent the larvae from escaping.
3. Allow the dish to stand at room temperature for 10–15 min during which time larvae 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 (**Fig. 2**).
4. Transfer only the larvae that have fed, each to a 1-oz plastic cup with a diet cube prepared as previously described. Cover the cup with a lid, place the cups on a plastic rack, and incubate at 28°C and 12:12 (L:D) light period.
5. To avoid and monitor possible contamination by viruses, it is advised to set up two mock treatments, one at the start and one at the end of treatment set up. On completion of treatments for one virus, use clean gloves for the next virus, and use a clean brush for transfer of larvae.
6. Bioassays should be replicated three or four times on separate occasions, with 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 once every 2–3 d.
8. Supply sufficient diet for each larva and continue monitoring until the mock-infected larvae (and any larvae that survive the virus treatments) have pupated.
9. Score the mortality and calculate  $\text{LC}_{50}$  values,  $\text{LC}_{50}$  95% confidence limits (CL), slope with standard error, and relative potency (or potency ratio) with 95% CL using an appropriate probit analysis program. Detailed procedures for these calculations are described in **Subheadings 3.6.1.** and **3.6.2.**

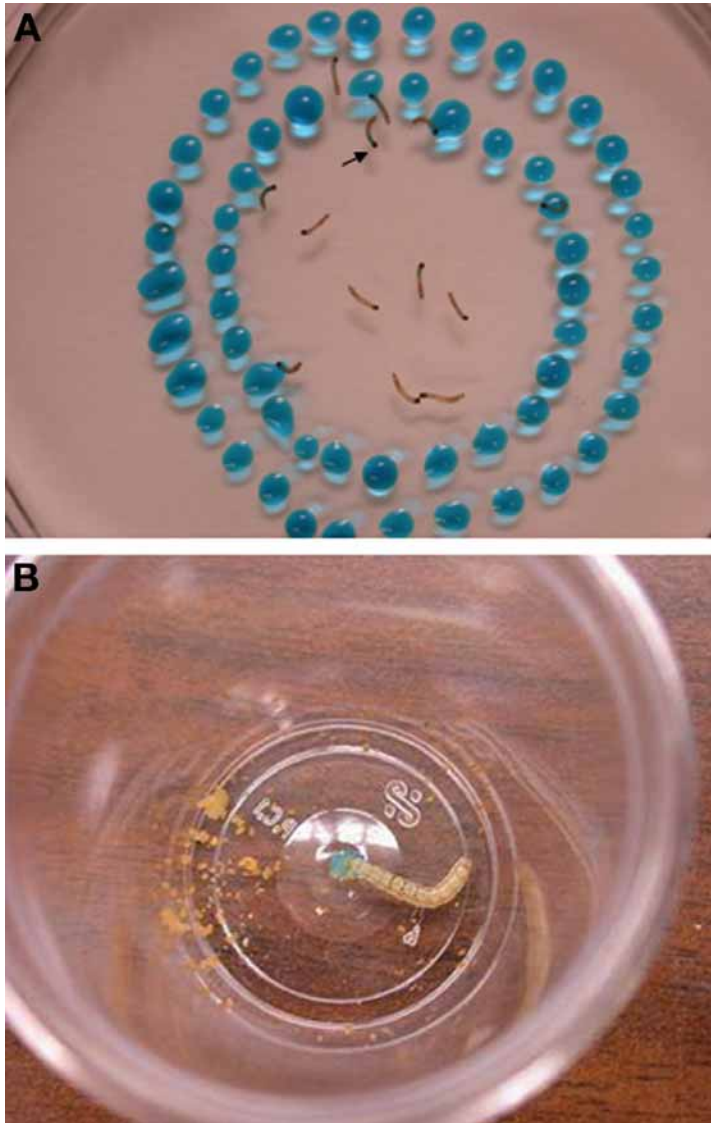


Fig. 2. Droplet and diet plug feeding bioassays. (A) Two concentric circles of droplets of virus solution and blue food dye are used for the droplet feeding bioassay. First, instar larvae placed in the center of the dish drink the virus solution and crawl up the sides of the dish. Larvae that have fed are identified by blue coloration of the gut (arrow). (B) Infection of third instar *Heliothis virescens* using the diet plug technique for determination of LD<sub>50</sub>. A known number of polyhedra are added to a small plug of diet, which the larva consumes over a period of 24 h. Only larvae that have completed consumed the diet are used for the bioassay.

### 3.3. Diet Plug Bioassay for Determination of Median Lethal Dose

1. The diet plug bioassay with polyhedral inclusion bodies is usually used for later instars (**Table 1**). Here, the authors describe the method for early fourth instars of *H. virescens*.
2. Prepare polyhedra as described for the droplet feeding bioassays. In diet plug bioassays, larvae are fed on a diet plug treated with a known dose of virus (**Fig. 2**). Five different concentrations are used with 30 larvae infected at each dose, and the bioassay replicated at least three times.
3. The specific virus doses required can 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<sup>3</sup>), transfer diet cubes into individual plastic cups (one cube per cup). If the diet cubes are too small they will dry out at low humidity.
5. Add 5 µL of polyhedra suspension with food coloring dye (4%, v/v) to each diet cube. Leave 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 excess clean diet. Starving larvae overnight beforehand will increase the speed with which larvae consume the diet cube (which typically takes around 3 h).
7. Maintain larvae at 28°C and a 12:12 (L:D) light period until the mock-infected larvae and the surviving larvae in the virus treatments have pupated, record dead larvae, and surviving insects.
8. Calculation of LD<sub>50</sub>, LD<sub>90</sub>, and 95% CL, slope with standard error, and relative potency (or potency ratio) with 95% CL is similar to that for LC<sub>50</sub> and LC<sub>90</sub> values (see **Subheadings 3.6.1.** and **3.6.2.**).

### 3.4. 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 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 (Burkard Scientific, Uxbridge, Middlesex, UK; **Fig. 3**). 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 (see **Note 4**) (**18**).

1. The titer of the working stock of budded virus should be determined (titring methodologies are given in Chapters 4, 5, 10, 11, and 21) and appropriate adjust-

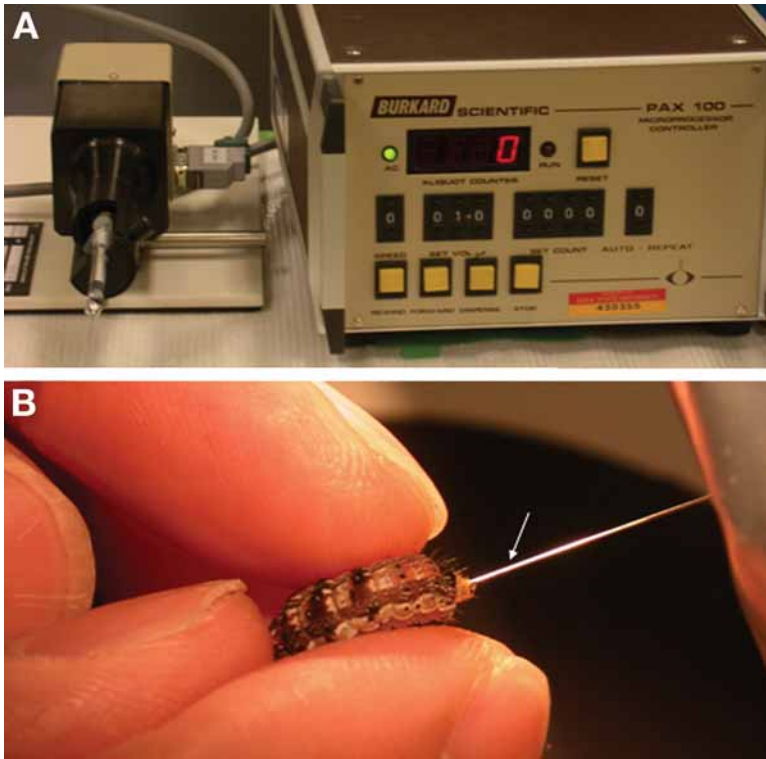


Fig. 3. Microapplicator assisted bioassays. (A) Microapplicator apparatus. The applicator with syringe mount and needle are shown on the left, the microprocessor control unit is on the right. (B) Oral inoculation of fifth instar *Heliothis virescens* using a 32-gauge blunt-tip needle as described in **Note 4**. This same apparatus can be used for injection of larvae with budded virus using a sharp-tip needle (see **Subheading 3.4**).

ments 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  $\mu\text{L}$  of BV solution (with a titer of  $10^7$ – $10^8$  pfu/mL) can be used for each larva, such that loading 300–500  $\mu\text{L}$  into the syringe is sufficient for most purposes.
3. Set the volume to 1.0  $\mu\text{L}$  for each droplet at the microprocessor control unit. Calibrate the control unit first to ensure that the correct volume is dispensed. The speed with which 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.

4. Drive air bubbles out of the syringe by holding the syringe upright to bring air to the needle tip and dispense. Then fix the syringe on to 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 prolegs and into the body cavity. Press the foot switch to deliver the BV solution into the hemocoel. To avoid piercing the gut tissue or body wall (see **Note 5**).
6. After injection, return all larvae to the 28°C incubator.
7. Check frequently for dead larvae and to ensure that adequate diet is available for surviving larvae. Late instars do not die from injury to the cuticle resulting from injection. Larvae should be checked 24 h after injection, however, in case of damage to the gut that could result in death from bacterial infection. This early mortality should be excluded from data analysis (see **Subheading 3.6.3.** for ST<sub>50</sub> data analysis).

### 3.5. 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 (2). However, assays performed on individual leaves may not be wholly representative of what occurs on a plant. If a larva is paralyzed by a neurotoxin for example, 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 (19).

1. For feeding assays, *H. virescens* second instar larvae are starved overnight and are mock-infected or infected with a 5X neonate LC<sub>99</sub> dose of AcMNPV-C6 and AcMLF9.ScathL by the droplet feeding method as described in **Subheading 3.2.2.** 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 iceberg lettuce on wet filter paper. Place the larvae in the growth chamber maintained at 28°C and 12:12 (L:D) light period.
3. Replace pieces of lettuce every 2–3 d.
4. Measure the areas of the pieces of lettuce with a LI-COR 3100 area meter (LI-COR Inc., Lincoln, NE) before and after feeding.
5. After all virus-infected larvae die, 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.

### 3.6. Data Analysis

For calculating the median lethal concentration (LC<sub>50</sub>) or median lethal dose (LD<sub>50</sub>) of a baculovirus, 95% confidence limits for the LC<sub>50</sub> or LD<sub>50</sub>, slope of

the dose-mortality response curve, and other related toxicological statistics, probit analysis is required using POLO-PC (7–9), or SAS (10). Use of the POLO-PC program and interpretation of the output are described in the following section.

### 3.6.1. Use of the POLO-PC Probit Analysis Program to Calculate $LC_{50}$ (or $LD_{50}$ )

1. Organize mortality data in an ASCII text file as shown below for data derived from the droplet feeding bioassay previously described. Treatment titles are preceded by an asterisk.

=Li's data (LC50 bioassays replicate 1)

```
*C6
0 30 0
0.5 29 6
2.0 30 20
5.0 28 21
10 30 28
20 30 30
*ScathL
0 30 0
0.5 29 12
2.0 30 17
5.0 29 23
10 30 29
20 30 30
```

The first column shows the concentration ( $\times 10^5$ ) of polyhedra, the second column shows the total number of treated insects in the treatment, and the third column shows the total number of dead larvae at the end of the bioassay.

2. Name the file, e.g., "LC-Hv," and save as a text file with the DOS suffix of ".txt:" "LC-Hv.txt." Save the data files in the same directory as the POLO.EXE program so that the POLO.EXE program will recognize them.
3. Start the POLO program either by double clicking the POLO.EXE icon in the file manager, or by typing "POLO" at either of these prompts:

```
C: \WINDOWS>POLO (return)
A:>POLO (return)
```

4. Once POLO has been started, the following screen should appear:

```
POLO PC
©Copyright LeOra software 1987
Input file>
```

5. At the POLO "Input file" prompt, type the data file name (LC-Hv.txt). Press return, and the data will appear on the screen.

6. Press return. A series of prompts will appear:
- Number of preparations = (number of separate trials carried out-POLO fills this in automatically);
  - Number of dose groups = (number of lines of numerical data-POLO fills this in);
  - Do you want probits (Y) =
  - Is natural response a parameter (Y) =
  - Do you want the likelihood function to be maximized (Y) =
  - LDs to calculate (10 50 90) =
  - Do you want to specify starting values of the parameters (N) =

The defaults to these questions are shown in brackets. Thus, unless special conditions apply, these questions can be answered by pressing return. Finally, all results are shown on the screen. To save and print the results, copy and paste them to a word file. For data derived from more than one replicate (*see Note 6*).

### 3.6.2. Interpretation of POLO Probit Analysis Output

1. An example of the POLO probit analysis output is provided in **Note 7**.
2. Test for linearity. The probit analysis assumes that a linear relationship exists between concentration (or dose) and response. If the relationship is not linear, then the analysis is not valid. The relationship is tested by determining whether the *t* ratio for slope (parameter estimation for slope/standard error) is greater than 1.96 (5% significant level for a *t* distribution with  $\times$  degrees of freedom). The *t* ratio (slope/standard error) must exceed 1.96 at the 5% level for the linear relationship to be significant. For our example, the POLO probit analysis output shows that *t* = 6.629 for C6 and 5.854 for ScathL. Because both *t* values >1.96, the probit-concentration relationship is linear.
3. Covariance matrix. This is used to estimate the confidence limits.
4. Chi-square test. If the chi-square is less than the critical chi-square value at  $p = 0.05$  and  $df = 3$  (check a chi-square table), then the data fit the probit model. For our example, chi-square = 2.553 for C6 and 4.615 for ScathL. Because both chi-square values are less than the critical value of 7.81, the data fit the probit model.
5. “*g*” test. If the chi-square value > the critical value, then there may be systematic deviation. In this case, you need to see if the index of significance for potency estimation (*g* value) < 0.5. If  $g < 0.5$ , then the data still fit the probit model, otherwise, the data do not fit the model, and the analysis is not valid. For ScathL, the  $g(.99)=1.5315$ , which is > 0.5, which means that the 99% CL cannot be estimated for lethal concentration values. The *g* values are generally lower for analysis of data from multiple replicates.
6. Estimations of lethal concentrations (or doses) and their confidence limits. First, determine if the two (or more if more treatments are involved) lines are the same (intercepts and slopes are equal, i.e., not significantly different) for both probit-dose lines. If the chi-square value is less than the chi-square at  $p = 0.05$  with  $df = 2$  (5.99), then accept the null hypothesis that the lines are the same. In our

**Table 2**  
**Dataset for a Lethal-Time Bioassay**

Virus 1 (trt 1)								
Hour post-infection (h pi)	86	90	95	102	110	118	123	138
Total larvae treated	23	23	23	23	23	23	23	23
Cumulative mortality	0	2	7	14	19	20	22	23
Virus 2 (trt 2)								
Hour postinfection (h pi)	86	90	95	102	110	118	123	138
Total larvae treated	23	23	23	23	23	23	23	23
Cumulative mortality	0	1	8	15	20	22	22	23

example (see output), the chi-square = 1.407 which is  $< 5.99$ , and the null hypothesis that the two lines are the same is accepted.

7. Test the hypothesis that the slopes are the same. If the chi-square statistic is less than the chi-square at  $p = 0.05$  with  $df = 1$  (3.84), then accept the null hypothesis that the slopes are the same. For our example, the chi-square = 0.671 which is  $< 3.84$ , and the null hypothesis is accepted.
8. Estimation of relative potency (potency ratio). This estimation is based on the assumption that the slopes for different treatments are the same. For example, the ScathL treatment appears to be 1.252 times more virulent than C6, but because the 0.90, 0.95, and 0.99 confidence limits cover the value 1.0, there is no significant difference in virulence between the two viruses (20).

### 3.6.3. Use of the S-Plus Kaplan-Meier Program to Calculate $ST_{50}$

Survival-time bioassays are performed using droplet feeding at an  $LC_{99}$  dose of virus (2), because the period of time taken by insects to consume the virus inoculum is relatively short using this method. Survival-time bioassays can also be performed using a microapplicator to deliver the budded virus derived from a polyhedrin-negative baculovirus directly into the larval hemocoel. Time is an important consideration for survival-time bioassays. After infection, mortality should be recorded every 4–8 h, with more frequent observations when the rate of mortality is high. Median survival times ( $ST_{50}$ ) and the 95% confidence limits are calculated using the Kaplan-Meier Estimator (11). Because the mortality data recorded at each time point for a group of insects are not independent observations, the  $ST_{50}$  cannot be calculated using probit analysis that is typically used for calculation of  $LC_{50}$  or  $LD_{50}$ . The comparison of multiple  $ST_{50}$ s is conducted using the log-rank test (11). The Kaplan-Meier procedure can be carried out using either a S-Plus or SAS program. A S-Plus Kaplan-Meier program is described here. Consider the dataset shown in Table 2 for a lethal-time bioassay.

1. Format the dataset as shown below and save as a .txt file (e.g., “demo.txt” in MySworck folder).

The datasets for virus 1 and 2 are shown below in two columns, but they should be set up as one continuous column. On each line of the column, the first number is the starting time point (h pi), the second is the ending time point (h pi), the third is for the treatment (e.g., “1” for Virus 1 [trt1] or “2” for Virus 2 [trt 2] for the example given in [Table 2](#)), and the fourth is 1, which represents the death of one larva between the two time points. If two or three larvae die during the same period, then repeat the appropriate data two or three times (i.e., the number of dead larvae = the number of rows).

86 90 1 1	86 90 2 1
86 90 1 1	90 95 2 1
90 95 1 1	90 95 2 1
90 95 1 1	90 95 2 1
90 95 1 1	90 95 2 1
90 95 1 1	90 95 2 1
90 95 1 1	90 95 2 1
95 102 1 1	90 95 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
95 102 1 1	95 102 2 1
102 110 1 1	95 102 2 1
102 110 1 1	102 110 2 1
102 110 1 1	102 110 2 1
102 110 1 1	102 110 2 1
102 110 1 1	102 110 2 1
102 110 1 1	102 110 2 1
110 118 1 1	102 110 2 1
118 123 1 1	110 118 2 1
118 123 1 1	110 118 2 1
123 138 1 1	123 138 2 1

2. Start up the Vincent%, at the prompt “Vincent%” type “add Splus,” press the “Enter” key, the prompt should be “>,”
3. Type the following commands line by line:

```
>colnames <- c("begin", "end", "trt", "status")
>data <- read.table("MySworck/demo.txt", col.names=colnames, header=F)
>data.trt <- as.factor(data$trt)
>mid <- (data$begin + data$end)/2
>data <- data.frame(data,mid)
>data.surv <- survfit (Surv(mid,status) ~trt, data = data)
>print (data.surv)
```

At this point, the following will appear on the screen:

Call: `survfit(formula = Surv(mid, status) ~ trt, data = data)`

	n	events	mean	se(mean)	median	0.95LCL	0.95UCL
trt=1	23	23	102	2.24	98.5	98.5	106
trt=2	23	23	101	1.97	98.5	92.5	106

The “median” in the output table shows the  $ST_{50}$  value for each virus,  $ST_{50}$  values are 98.5 (h), the 95% CL are 98.5–106 (h) for virus 1 and 92.5–106 (h) for virus 2.

Type: `>summary (data.surv)` (press “Enter”), for additional detail as shown next:

Call: `survfit(formula = Surv(mid, status) ~ trt, data = data)`

trt=1						lower	upper
time	n.risk	n.event	survival	std.err	95% CI	95% CI	
88.0	23	2	0.9130	0.0588	0.80485	1.000	
92.5	21	5	0.6957	0.0959	0.53088	0.912	
98.5	16	7	0.3913	0.1018	0.23504	0.651	
106.0	9	5	0.1739	0.0790	0.07137	0.424	
114.0	4	1	0.1304	0.0702	0.04541	0.375	
120.5	3	2	0.0435	0.0425	0.00639	0.296	
130.5	1	1	0.0000	NA	NA	NA	

trt=2						lower	upper
time	n.risk	n.event	survival	std.err	95% CI	95% CI	
88.0	23	1	0.9565	0.0425	0.87671	1.000	
92.5	22	7	0.6522	0.0993	0.48389	0.879	
98.5	15	7	0.3478	0.0993	0.19876	0.609	
106.0	8	5	0.1304	0.0702	0.04541	0.375	
114.0	3	2	0.0435	0.0425	0.00639	0.296	
130.5	1	1	0.0000	NA	NA	NA	

- To determine whether there is a significant difference in survival time of the two viruses, type: `>survdif (Surv[mid,status] ~trt, data = data)` (press “Enter”), and the results will appear as follows.

Call: `survdif(formula = Surv(mid, status) ~ trt, data = data)`

	N	Observed	Expected	(O-E)^2/E	(O-E)^2/V
trt=1	23	23	24.1	0.0497	0.178
trt=2	23	23	21.9	0.0547	0.178

Chisq= 0.2 on 1 degrees of freedom, p= 0.673

The  $p = 0.673$ , shows that there is no significant difference in survival time of the two viruses. If there are more than two treatments to be compared, then you will need two or more txt files with different file names, with results for two data sets only in each file. Run the program using each pair-wise dataset.

#### 4. Notes

1. Before molting, larvae usually do not eat and are quiescent. 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, old head capsule and the black band on the thorax. Larvae will molt and shed the old cuticle within several hours of when this white band appears at 28°C. If you want to stop the molt while waiting for other larvae to reach the premolt stage, then you can put them at 4°C. When you have sufficient larvae, return them to 28°C, and check frequently. You will see some larvae shedding their old cuticle and some that have already molted. 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 (18).
2. Concentrated sodium azide (10% stock solution) is poisonous, and should be handled carefully with gloves. The sodium azide is diluted sufficiently during preparation of polyhedra for bioassay treatments that it will not affect the larvae.
3. If larvae are too crowded, then they may eat each other. The number of larvae should not exceed 20 individuals per dish.
4. 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. 3). Set the syringe on the support tube of the microapplicator. Under a microscope, hold a larva (such as early fourth or fifth instar of *H. virescens*), insert the needle through the mouth and into the anterior region of the larval midgut where polyhedra are delivered. This technique requires some practice.
5. 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. If the data from three or more replicates need to be processed to calculate LC<sub>50</sub> or LD<sub>50</sub>, then arrange the data for multiple replicates in the same file.
7. Example of POLO Probit Analysis Output:

POLO-PC

(C) Copyright LeOra Software 1987

Input file > LC-Hv.txt

input: =3/1/05-Li's data (LC50 bioassays replication 1)

input: \*C6

input: 0 30 0

input: 0.5 29 6

input: 2.0 30 20

input: 5.0 28 21  
input: 10 30 28  
input: 20 30 30  
input: \*ScathL  
input: 0 30 0  
input: 0.5 29 12  
input: 2.0 30 17  
input: 5.0 29 23  
input: 10 30 29  
input: 20 30 30

preparation	dose	log-dose	subjects	responses	resp/subj
C6	.00000	.000000	30.	0.	.000
	.50000	-.301030	29.	6.	.207
	2.00000	.301030	30.	20.	.667
	5.00000	.698970	28.	21.	.750
	10.00000	1.000000	30.	28.	.933
	20.00000	1.301030	30.	30.	1.000
ScathL	.00000	.000000	30.	0.	.000
	.50000	-.301030	29.	12.	.414
	2.00000	.301030	30.	17.	.567
	5.00000	.698970	29.	23.	.793
	10.00000	1.000000	30.	29.	.967
	20.00000	1.301030	30.	30.	1.000

Number of preparations: 2

Number of dose groups: 10

Do you want probits [Y] ?

Is Natural Response a parameter [Y] ?

Do you want the likelihood function to be maximized [Y] ?

LD's to calculate [10 50 90] >

Do you want to specify starting values of the parameters [N] ?

The probit transformation is to be used

The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is (1) C6

Not estimating natural response

Maximum log-likelihood -58.491284

	parameter	standard error	t ratio
C6	-.26632344	.16878642	-1.5778724
SLOPE	1.7694434	.26692043	6.6291045

Variance-Covariance matrix

	C6	SLOPE
C6	.2848885E-01	-.2820615E-01
SLOPE	-.2820615E-01	.7124652E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
C6	29.	6.	6.152	-.152	.212151
	30.	20.	18.150	1.850	.605008
	28.	21.	23.355	-2.355	.834092
	30.	28.	28.008	-.008	.933596
	30.	30.	29.373	.627	.979114

chi-square 2.5529 degrees of freedom 3 heterogeneity .85

Index of significance for potency estimation:

g(.90)=.06157 g(.95)=.08742 g(.99)=.15098

Effective Doses

	dose	limits	0.90	0.95	0.99
LD10 C6	.26684	lower	.11935	.09641	.05804
		upper	.44507	.48152	.55448
LD50 C6	1.41421	lower	.98271	.90144	.74226
		upper	1.88973	1.99104	2.20393
LD90 C6	7.49516	lower	5.33777	5.04693	4.54811
		upper	12.16289	13.74920	18.28318

Intercepts and slopes unconstrained. Preparation is (2) ScathL

Not estimating natural response

Maximum log-likelihood -62.076102

	parameter	standard error	t ratio
ScathL	.48471674E-02	.15753428	.30768969E-01
SLOPE	1.4698506	.25108058	5.8540993

Variance-Covariance matrix

	ScathL	SLOPE
ScathL	.2481705E-01	-.2293147E-01
SLOPE	-.2293147E-01	.6304146E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
ScathL	29.	12.	9.594	2.406	.330830
	30.	17.	20.180	-3.180	.672677
	29.	23.	24.622	-1.622	.849017
	30.	29.	27.896	1.104	.929853
	30.	30.	29.172	.828	.972392

chi-square 4.6152 degrees of freedom 3 heterogeneity 1.5384

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted.

See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:

$g(.90)=.24862$   $g(.95)=.45464$   $g(.99)=1.5315$

"With almost all good sets of data,  $g$  will be substantially smaller than 1.0, and seldom greater than 0.4."

-D. J. Finney, "Probit Analysis" (1972), page 79.

Effective Doses

	dose	limits	0.90	0.95	0.99
LD10 ScathL	.13329	lower	.00695	.00031	
		upper	.38729	.49352	
LD50 ScathL	.99244	lower	.31147	.10957	
		upper	1.80757	2.19647	
LD90 ScathL	7.38915	lower	3.88929	3.23634	
		upper	30.29740	117.18609	

Intercepts and slopes constrained (lines are the same)

Not estimating natural response

Maximum log-likelihood -121.27105

	parameter	standard error	t ratio
INTERCPT	-.12327583	.11437667	-1.0778058
SLOPE	1.6094219	.18174230	8.8555163

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.1308202E-01	-.1243525E-01
SLOPE	-.1243525E-01	.3303026E-01

Testing hypothesis that slopes and intercepts are the same  
chi-square 1.4073 degrees of freedom 2 tail probability .495

Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
INTERCPT	29.	6.	7.879	-1.879	.271673
	30.	20.	19.231	.769	.641028
	28.	21.	23.569	-2.569	.841747
	30.	28.	27.941	.059	.931380
	30.	30.	29.269	.731	.975617
	29.	12.	7.879	4.121	.271673
	30.	17.	19.231	-2.231	.641028
	29.	23.	24.411	-1.411	.841747
	30.	29.	27.941	1.059	.931380
	30.	30.	29.269	.731	.975617

chi-square 8.7521 degrees of freedom 8 heterogeneity 1.0940

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:  
 $g(.90)=.04824$   $g(.95)=.07418$   $g(.99)=.15707$

Effective Doses

	dose	limits	0.90	0.95	0.99
LD10 INTERCPT	.19068	lower	.08861	.06915	.03286
		upper	.31771	.35087	.43204
LD50 INTERCPT	1.19288	lower	.84807	.76692	.57724
		upper	1.56445	1.66105	1.90581
LD90 INTERCPT	7.46237	lower	5.45446	5.11015	4.42812
		upper	11.46363	13.08797	19.25410

Slopes constrained (lines are parallel)  
 Not estimating natural response

Maximum log-likelihood -120.90265

	parameter	standard error	t ratio
C6	-.20475452	.14911421	-1.3731389
ScathL	-.47043748E-01	.14495005	-.32455143
SLOPE	1.6163655	.18267648	8.8482413

Variance-Covariance matrix

	C6	ScathL	SLOPE
C6	.2223505E-01	.4699631E-02	-.1363601E-01
ScathL	.4699631E-02	.2101052E-01	-.1150116E-01
SLOPE	-.1363601E-01	-.1150116E-01	.3337070E-01

Testing hypothesis that slopes are the same  
 chi-square .6705 degrees of freedom 1 tail probability .413  
 Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparations	subjects	responses	expected	deviation	probability
C6	29.	6.	7.096	-1.096	.244679
	30.	20.	18.329	1.671	.610959
	28.	21.	23.031	-2.031	.822527
	30.	28.	27.629	.371	.920968
	30.	30.	29.135	.865	.971164
ScathL	29.	12.	8.607	3.393	.296803
	30.	17.	20.096	-3.096	.669862
	29.	23.	24.956	-1.956	.860540
	30.	29.	28.251	.749	.941714
	30.	30.	29.403	.597	.980104

chi-square 7.9730 degrees of freedom 7 heterogeneity 1.1390

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:  
 g(.90)=.05222 g(.95)=.08135 g(.99)=.17816

Effective Doses

	dose	limits	0.90	0.95	0.99
LD10 C6	.21568	lower	.09267	.06986	.02872
		upper	.38219	.42960	.55391
ScathL	.17228	lower	.07238	.05414	.02170
		upper	.30954	.34885	.45223
LD50 C6	1.33868	lower	.85484	.74807	.50826
		upper	1.95163	2.13539	2.66086
ScathL	1.06931	lower	.67222	.58480	.38954
		upper	1.57011	1.71919	2.14211
LD90 C6	8.30875	lower	5.50908	5.02786	4.06871
		upper	14.26568	16.91105	28.25755
ScathL	6.63689	lower	4.41387	4.02729	3.25173
		upper	11.26439	13.28765	21.81563

Relative potencies

	potency	limits	0.90	0.95	0.99
ScathL	1.25190	lower	.73007	.63283	.43308
		upper	2.16510	2.51078	3.74228

3/1/05-Li's data (LC50 bioassays replication 1)  
 C6 subjects 147 controls 30  
 $\log(L)=-58.49$  slope= $1.769\pm.267$  nat.resp.=.000 $\pm$ .000  
 heterogeneity=.85 g=.087  
 LD10=.267 limits: .096 to .482  
 LD50=1.414 limits: .901 to 1.991  
 LD90=7.495 limits: 5.047 to 13.749  
 ScathL subjects 148 controls 30  
 $\log(L)=-62.08$  slope= $1.470\pm.251$  nat.resp.=.000 $\pm$ .000  
 heterogeneity=1.54 g=.455  
 LD10=.133 limits: .000 to .494  
 LD50=.992 limits: .110 to 2.196  
 LD90=7.389 limits: 3.236 to 117.186  
 SAME subjects 295 controls 60  
 $\log(L)=-121.3$  slope= $1.609\pm.182$  nat.resp.=.000 $\pm$ .000  
 Hypothesis ACCEPTED  
 heterogeneity=1.09 g=.074  
 LD10=.191 limits: .069 to .351  
 LD50=1.193 limits: .767 to 1.661  
 LD90=7.462 limits: 5.110 to 13.088  
 PARALLEL subjects 295 controls 60  
 $\log(L)=-120.9$  slope= $1.616\pm.183$  nat.resp.=.000 $\pm$ .000  
 Hypothesis ACCEPTED  
 heterogeneity=1.14 g=.081  
 LD10=.216 limits: .070 to .430  
 LD50=1.339 limits: .748 to 2.135  
 LD90=8.309 limits: 5.028 to 16.911  
 Stop - Program terminated.

## Acknowledgments

The authors would like to thank Drs. Loy Volkman and Jan Washburn for training HL in accurate staging of larvae and use of the microapplicator, and Dr. Robert Harrison for honing of some of the methods described herein. This material is based upon work supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Agreement no. 2003-35302-13558 as well as Hatch Act and State of Iowa funds.

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## Monitoring and Visualization of Baculovirus Infection Using Green Fluorescent Protein Strategy

Hyung Joon Cha and William E. Bentley

### Summary

Through green fluorescent protein (GFP) strategy, facile and fast monitoring and visualization of baculovirus infection in insect cells is possible *in vivo*. This chapter describes two novel techniques for simple determination of virus titer in the baculovirus expression system using GFP coexpression and rapid monitoring of infection of Sf-9 insect cells using combination of GFP and early-to-late (ETL) promoter. It is anticipated that the use of GFP 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) per milliliter, when preparing new virus stocks or when carrying out infections for protein production. The productivity of the baculovirus expression vector system is sensitive to cell density, viability, nutrient levels, and the multiplicity of infection, which is the ratio of added virus (pfu) 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 proteins. 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*); Chapter 4) or by end-point dilution (*1*);

Chapter 10). (Other methods of titering a baculovirus stock are given in Chapters 5 and 11.) Although the plaque assay has the potential to accurately determine the virus titer, it is difficult to perform and requires a long processing time (approx 1 wk). Furthermore, visualizing plaques can be difficult. Although the end-point dilution method is easier to setup, the results are often more difficult to interpret than plaque assays, particularly when titrating recombinant virus stocks. Wild-type virus is easy to detect because of the accumulation of 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 are monitored (with difficulty) by ordinary light microscopy. To overcome this problem, the galactosidase  $\beta$ -(*gal*) gene (*lacZ*) was introduced into the transfer vectors (3). Therefore, recently, many commercially available transfer vectors contain this infection marker gene. However, the use of this marker requires an additional expensive substrate (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside [X-gal]), which must be added in an additional step near the end of the assay. By incorporating the gene for the 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 was originally isolated from the jellyfish, *Aequorea victoria*, and was first described in 1962 (4). 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 from the organism to GFP (5). The cDNA of GFP was cloned from *A. victoria* in 1992 (6). GFP is a 238-amino acid protein with a molecular weight of 27 kDa (7). GFP has a major absorption peak at 395 nm and a minor peak at 470 nm with a single emission peak at 509 nm (7). 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 require fixation techniques that are toxic to the cells under investigation (8). As such, GFP has been studied recently as a novel genetic reporter molecule (see Note 1).

The baculovirus expression vector system is both effective and convenient for the overproduction of recombinant proteins in eukaryotic cells (9–11). The strong polyhedrin (*polh*) promoter (12,13) and the 10-kDa fibrous polypeptide (*p10*) promoter (11,14), 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 more than 50% of the total protein (15). The use of these promoters to establish the progress of infection and protein production requires waiting as long as 48 h postinfection (*pi*). Crawford and Miller (16) characterized the role of several early baculovirus genes on the expression of late viral genes. They also noted the  $\beta$ -galactosidase produced under the early-to-late (ETL)

promoter could be useful in aiding the identification of occlusion-body negative recombinants. Visualization of the infection process can be particularly difficult. The availability of a reporter protein can greatly facilitate developmental work (3,17,18). Richardson et al. (17) developed a method that uses the  $\beta$ -galactosidase under control of early and late promoters, including the ETL promoter, to help aid in plaque assays. Coupling the advantages of ETL promoter with GFP can endow a simpler and early monitoring of baculovirus infection.

## 2. Materials

### 2.1. Cell Culture and Medium

1. Insect cells (*Spodoptera frugiperda*, Sf-9) are obtained from American Type Culture Collection (ATCC, cat. no. CRL 1711).
2. TNM-FH insect medium (JRH Biosciences) supplemented with 10% fetal bovine serum (Sigma) or SF900-II serum-free medium (Invitrogen).
3. 0.4% Trypan blue solution (Sigma).
4. Hemacytometer (Fisher Scientific).
5. 60-mm Tissue-culture dish (Falcon).
6. 25-mL Tissue-culture flask (Falcon).

### 2.2. Expression Vector and Baculovirus

1. The vector pGFPuv (Clontech).
2. The transfer vector pVL1392 (PharMingen).
3. The transfer vector pBlueBacHis2/CAT (Invitrogen).
4. The recombinant transfer vector pVLGFPuv, obtained from pVL1392 and pGFPuv (19).
5. The recombinant transfer vector pBBH( $\Delta$ lacZ)GFPuv, obtained from pBlueBacHis2/CAT and pGFPuv (20).
6. The recombinant transfer vector pBBH-GFPuv/CAT, obtained from pBlueBacHis2/CAT and pGFPuv (20).
7. Linearized BaculoGold™ Wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) DNA (PharMingen).
8. Bac-N-Blue™ wild-type AcMNPV DNA (Invitrogen).
9. The recombinant baculovirus,  $\nu$ GFPuv, obtained by cotransfecting Sf-9 cells with pVLGFPuv and linearized BaculoGold wild-type AcMNPV DNA.
10. The recombinant baculovirus,  $\nu$ P<sub>ETL</sub>-GFPuv, obtained by cotransfecting Sf-9 cells with pBBH( $\Delta$ lacZ)GFPuv and Bac-N-Blue wild-type AcMNPV DNA.
11. The recombinant baculovirus,  $\nu$ PH-GFPuv/CAT, obtained by cotransfecting Sf-9 cells with pBBH-GFPuv/CAT and Bac-N-Blue wild-type AcMNPV DNA.
12. Phosphate buffered saline (PBS) buffer (Sigma).
13. UV transilluminator (Cole Parmer).
14. 96-Well plate (Nunc).
15. Fluorescence spectrometer (Perkin-Elmer).
16. Fluorescent microscopy (Olympus).

### 2.3. Western Blotting for GFP

1. Sample buffer (5X): 125 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v)  $\beta$ -mercaptoethanol, and 0.25% (w/v) bromophenol blue. Store at room temperature.
2. Stacking buffer (4X): 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS. Store at 4°C.
3. Running buffer (4X): 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; Bio-Rad).
7. 10% (w/v) Ammonium persulfate: prepare solution in water and immediately freeze in single use (200  $\mu$ L) aliquots at -20°C.
8. Water-saturated isobutanol: shake equal volumes of water and isobutanol in a glass bottle and allow to separate. Use the top layer. Store at room temperature.
9. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad). Load 10  $\mu$ L/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-20 (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 (Sigma).
20. Supported nitrocellulose membrane (Millipore).
21. 3 MM chromatography paper (Whatman).

## 3. Methods

### 3.1. Construction of Recombinant Expression Vectors and Establishment of Recombinant Baculoviruses

1. Excise the *gfp<sub>uv</sub>* gene from the pGFP<sub>uv</sub> plasmid using *Pst*I and *Eco*RI digestion.
2. Insert the *gfp<sub>uv</sub>* gene (from **step 1**) under the polh promoter of the pVL1392 baculovirus transfer vector to obtain the pVLGFP<sub>uv</sub> transfer vector (**19**), which is subsequently amplified in *Escherichia coli* (see **Note 3**).

3. Cotransfect  $2 \times 10^6$  Sf-9 cells with the 5- $\mu$ g pVLGFPuv transfer vector (from **step 2**) and 0.5  $\mu$ g linearized BaculoGold wild-type AcMNPV DNA to obtain the vGFPuv recombinant baculovirus (*see Note 4*).
4. Construct pBlueBacHis2( $\Delta$ lacZ) transfer vector (**20**) by PCR amplification around the *lacZ* gene sequence of the pBlueBacHis2/CAT transfer vector using the primer sets (5'-gctctagattttagcagtgattctaattgcagc-3' and 5'-ccgcgccgccaccacacgcccgaaccagctac-3', 5'-ggcgccgccactctcagtaacaatctgctctg-3' and 5'-gctctagacggccgctgaggccgatactgtctgctgc-3').
5. Excise the *gfp<sub>uv</sub>* gene from the pGFPuv plasmid using *EagI* and *XbaI* digestion.
6. Insert the *gfp<sub>uv</sub>* gene (from **step 5**) in frame under the ETL promoter of the pBlueBacHis2( $\Delta$ lacZ) transfer vector (from **step 4**) resulting in a transfer vector denoted pBBH( $\Delta$ lacZ)GFPuv.
7. Cotransfect  $2 \times 10^6$  Sf-9 cells with the 5  $\mu$ g pBBH( $\Delta$ lacZ)GFPuv transfer vector (from **step 6**) and 0.5  $\mu$ g Bac-N-Blue wild-type AcMNPV DNA to obtain the vP<sub>ETL</sub>-GFPuv recombinant baculovirus.
8. Amplify the *gfp<sub>uv</sub>* gene from the pGFPuv plasmid using the PCR (primers: 5'-GGCT AGCATGAGTAAAGGAGAAGAAGACTTTTC-3' and 5'-GGCTAGCTTTGTAGAGCTCATCCATGCC-3').
9. Digest the PCR-amplified *gfp<sub>uv</sub>* gene using *NheI*.
10. Insert the *gfp<sub>uv</sub>* 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 (**20**) 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  $\mu$ g pBBH-GFPuv/CAT transfer vector (from **step 10**) and 0.5  $\mu$ g Bac-N-Blue wild-type AcMNPV DNA to obtain the vPH-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-mL tissue-culture flasks at 27°C.
13. Grow Sf-9 cells in SF900-II SFM medium and subculture routinely every 3–4 d (**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 modification of the end-point dilution (*see* Chapter 10) by incorporating a GFP marker for the simple determination of virus stock titer (*see Note 5*). Because GFP imposes minimal metabolic burden on the host cells, we anticipate that the use of GFPuv as a fusion partner or coexpressed with a desired foreign protein (under different promoters) will facilitate recombinant baculovirus titering in a nonintrusive manner.

1. Dilute the virus stock samples serially  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$ .
2. Mix 10- $\mu$ L aliquots of each virus dilution with 90- $\mu$ L aliquots of a Sf-9 cell suspension from the exponential growth phase to obtain a total volume of 100  $\mu$ L (dilute to  $10^5$  cells/mL).

3. Use each of the 100- $\mu$ L virus/cell samples obtained in **step 2** to seed 12 wells of four 96-well plates (*see Note 6*).
4. Incubate the plates obtained in **step 3** at 27°C for 4 d.
5. Check each well for virus infection on an UV table. Green light, which is because of GFP expression, indicates virus infection and thus a positive well (*see Note 7*).
6. Calculate the titer (pfu/mL) of the recombinant baculovirus  $\nu$ 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 ( $\sim 10^6$  cells/mL) with a predetermined volume of the  $\nu P_{\text{ETL}}$ -GFPuv and  $\nu$ PH-GFPuv/CAT recombinant baculoviruses to yield a multiplicity of infection of five (*see Chapter 1 and Notes 9 and 10*).
2. Collect culture samples initially every 6 h for the first 60 h pi 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 postinfection using samples collected in **step 2**.
6. Prepare a 1.5-mm thick, 10% gel by mixing 7.5 mL of 4X separating buffer, with 10 mL acrylamide/bis solution, 12.5 mL water, 100  $\mu$ L ammonium persulfate solution, and 20  $\mu$ L 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 4X stacking buffer with 1.3 mL acrylamide/bis solution, 6.1 mL water, 50  $\mu$ L ammonium persulfate solution, and 10  $\mu$ L 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 4X running buffer with 400 mL of water in a measuring cylinder. Cover with Parafilm 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 the 50  $\mu$ L 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 approx 30–40 mA through the separating gel.

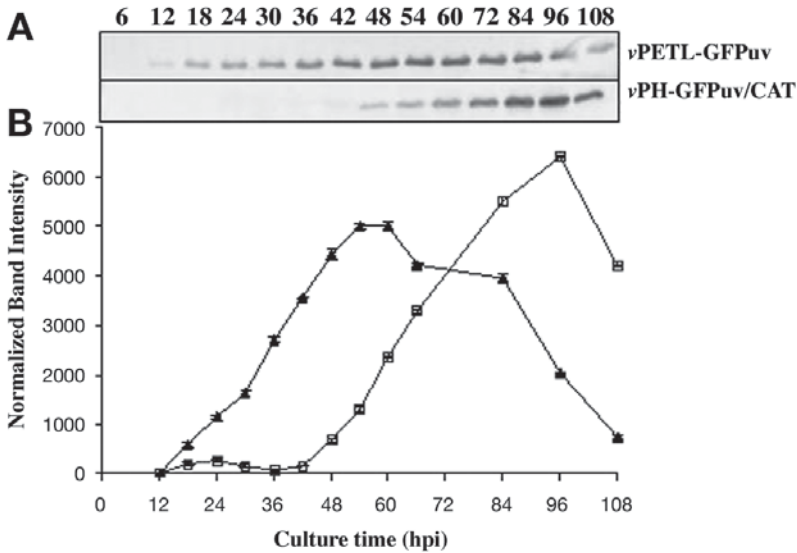


Fig. 1. Western blot analyses of green fluorescent protein expression under (ETL) (▲) and polh (□) promoters. (A) Developed Western blots. (B) Quantified results that were normalized to the first non-zero intensities from scanned images (12 h pi, ETL signal).

13. Transfer the samples that have been separated by SDS-polyacrylamide gels onto supported nitrocellulose membranes electrophoretically (*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.

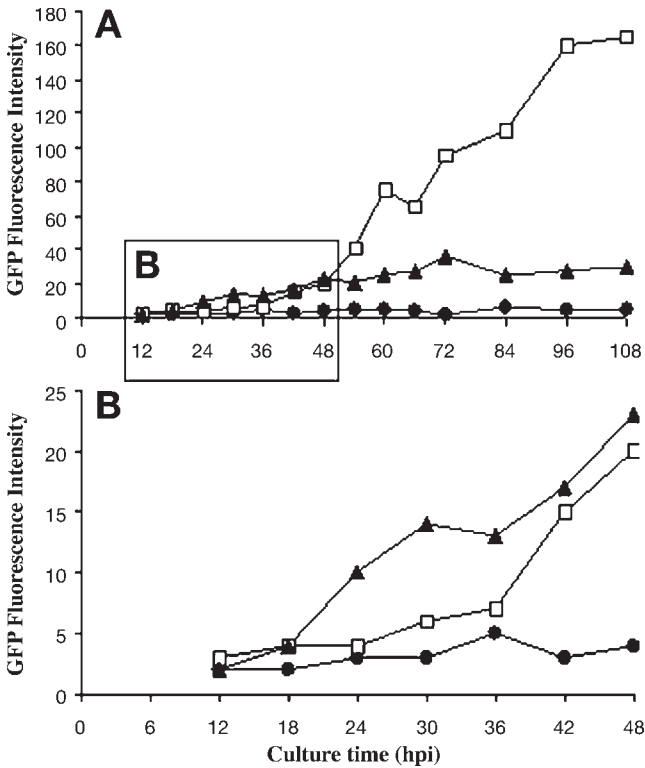


Fig. 2. Green fluorescent protein (GFP) fluorescence intensity for control (●) and cultures infected with vP<sub>ETL</sub>-GFPuv (▲) and vPH-GFPuv/CAT (□). (A) Fluorescence intensity over entire culture time. (B) Fluorescence intensity for first 48 h pi.

#### 4. Notes

1. In this research, we can use a GFP variant, GFPuv, which is optimized for UV excitation (8). 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.
2. This is a neurotoxin when unpolymerized and so care should be taken not to receive exposure.
3. This strategy leaves available the p10 promoter (another strong baculovirus promoter) for expression of genes of interest from the same virus.
4. Successful cotransfection 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 d for end-point dilution and 6–7 d 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. 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 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-d 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 polh 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 approx 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 pi, after which the accumulation level declines steadily. The decrease is possibly due to proteolysis (21) 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 approx 42 h pi with a maximum at 96 h pi followed by a sharp decrease. These results correspond well to the expected profile, where immediate-early and delayed-early genes are expressed between approx 0 and 10 h pi, the transition to the late phase occurring between 10 and 15 h pi, and the very late genes express after 18 h pi (22).
13. Consistent with Western blots, the GFP fluorescence intensity under the polh promoter increases rapidly after 42 h pi until reaching a maximum at approx 96 h pi, but without a marked decline. Under the early ETL promoter, GFP fluorescence remains low and fairly constant after 48 h pi, but increases initially near 18 h pi (see insert in Fig. 2B) resulting in a similar 18 h time shift observed by Western analysis.

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## Alternative Bioreactor Strategy for Probing Infection and Production

Yu-Chen Hu and William E. Bentley

### Summary

Probing the baculovirus infection process is essential in optimizing the recombinant protein production. Typically, researchers monitor the infection process in the stirred tank reactor, which, however, contains a population of cells infected at different times after virus inoculation. This chapter describes 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 (which utilizes host insect cells) has been widely used for protein production. To grow insect cells and produce proteins of interest, a variety of stirred tank bioreactors are commercially available and some novel reactor systems, e.g., packed-bed reactors (*1*), wave bioreactors (*see* Chapter 12) and two-stage continuous stirred tank reactors (CSTR), where the first CSTR is used for cell growth and the second for viral infection and production (*2*) have been proposed. One key to optimal protein production is the selection of optimal infection strategies. Although various models relating the multiplicity of infection (MOI; effectively, the infectious virus particle/cell ratio) to the product yield have been proposed (*3–5*), it is generally difficult to interpret these models in a more segregated way and to monitor 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 reactor or spinner flask. For example, after virus addition to the stirred tank reactor, some cells may be infected immediately whereas some cells may be infected several hours later. The proportion of cells that are not immediately infected gets higher as the MOI is reduced (e.g., below five). The analysis of cells at a *certain* time postinfection, therefore, actually is the analysis of a *population of cells in a period of time postinfection* and may lead to misinterpretation of results. To circumvent this problem, a two-stage bioreactor system consisting of an upstream CSTR that provides cells continuously to a downstream tubular reactor with segmented plug flow for viral infection is reported (6). 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 time (i.e., the time required by the liquid element to move from the entrance to the exit of the tubular reactor). By taking samples in the liquid segment at different distances down the tubular reactor (i.e., different times postinfection), 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 (JRH Biosciences) is supplemented with 0.1% (w/v) Pluronic® F-68 (JRH Biosciences), 100 mg/L ampicillin (Sigma) (*see Note 1*), 2.5 g/mL fungazone (Advanced Biotechnologies, Inc.), 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: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.4 mM KH<sub>2</sub>PO<sub>4</sub>.
5. Recombinant baculovirus expressing the reporter protein. In this case, vIBD-7 (7), a recombinant baculovirus expressing galactosidase β-(gal), is used.
6. O-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) for β-gal activity assay.

### 2.2. Two-Stage Bioreactor System

1. Air-conditioned culture room controlled at 27–28°C.
2. A glass-blown jacketed spinner flask (working volume = 200 mL) made in house.
3. 1-L medium bottles with multiport cap assemblies (Bellco Biotechnology) to serve as medium reservoir and waste jar.
4. Stirrer plate (Bellco Biotechnology).
5. Water bath (VWR International).
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 four-channel pump head (model 205BA).
11. Watson-Marlow silicone tubing (ID = 1.14 mm) and MasterFlex 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 an air-conditioned 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 (agitated speed = 80 rpm).
3. When the cells enter the exponential phase ( $\sim$ (approx  $7\text{--}7.5 \times 10^5$  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  $0.0315 \text{ h}^{-1}$ .
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. The mixing creates a segmented flow pattern (**Fig. 1**, *see Note 4*) whose validity has been demonstrated previously (**6**).
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 declamped and then pump 4 is turned on again. The infected cells are washed with phosphate buffered-saline, 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  $\beta$ -gal activities are measured (**8**). If recombinant baculovirus expressing other reporter protein (e.g., green fluorescent protein, *see Chapter 21*) 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 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  $\beta$ -gal expression at different residence times. **Figure 2** shows a lower  $\beta$ -gal yield at residence times = 1.6 h (MOI = 60, *see Note 7*), indicating that the infection is incomplete. At higher residence times (3.2 and 5.1 h), there are no significant differences in either the rate of increase or the level of  $\beta$ -gal activity, indicating that at MOI = 60 the infection process is completed prior to 3.2 h postinfection (*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 because insect cells grow optimally at 27–28°C. Furthermore, the pH is not controlled because the insect cell medium has a strong buffer capacity and does not change significantly under these operating conditions.
3. Because 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.

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Fig. 1. (*opposite page*) 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 ( $\approx 2$  mm ID) is annealed to the CSTR and protrudes from the side of the reactor at 45° angle downward from the vertical, and connected via a Watson-Marlow silicone tubing (ID = 1.14 mm) to the tubular reactor entrance, thus allowing for the cells to flow out to the tubular reactor. The tubular reactor is comprised of a multi-channel pump (Watson Marlow, model 505Du) and a semi-permeable silicone tube (MasterFlex Masterflex no. 14, length = 7.6 m, ID = 1.6 mm). The pump accommodates a four-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.

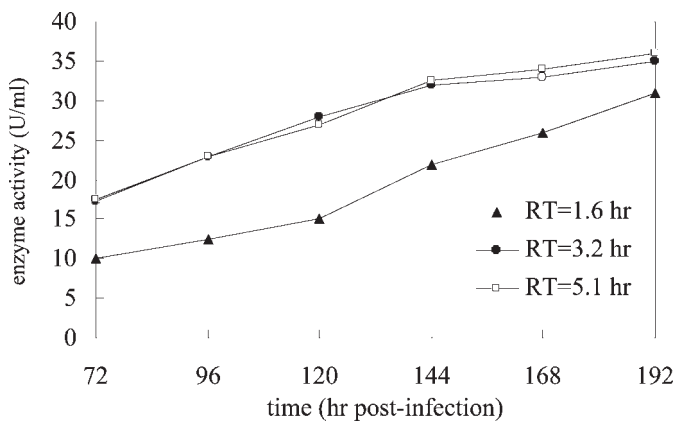


Fig. 2. Time-course profile of  $\beta$ -gal activity in cells infected for different residence times (RT) at MOI = 60. Cells are collected at the exit of the tubular reactor operated at different RT, washed in phosphate buffered-saline and seeded to T-25 flasks for expression.

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 hydraulic 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 postinfection 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 MOIs, 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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## Methods for Gene Silencing With RNAi

John C. March and William E. Bentley

### Summary

This chapter describes a technique for synthesizing and transfecting double stranded RNA (dsRNA) for RNA interference in Sf-21 cell culture. Transfection with dsRNA only requires 1 h and the cells are usually recovered 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<sup>®</sup>-4PCR and MEGAscript<sup>™</sup> T7 kit from Ambion and the Superscript<sup>™</sup> III kit from Invitrogen.

**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 knock-out strains. Further, 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-21 (1) and Sf-9 (unpublished data from our lab). The strategies for silencing genes in insect cell culture have mostly focused on in vitro-synthesized double stranded RNA (dsRNA). In these studies, dsRNA was synthesized enzymatically in vitro and transfected into the cells (2). dsRNA silencing of both viral (3,4) and host (5) genes has been demonstrated. The use of RNAi allowed for transient inhibition of several genes that were expected to increase titer or product yield (3–5). The strategy for each is the same. More recently, it has been

shown that baculovirus can be used to *deliver* short interfering RNA (siRNA) to a host for *in vivo* transcription and silencing (6) (see **Note 1**). The method described here is a modified version (submitted manuscript) of that reported by Clemens et al. (2) for *in vitro* dsRNA synthesis and transfection into Sf-9 cells.

## 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 (7). 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 and coworkers (8). It is highly recommended that off-target effects are considered in designing dsRNA for gene silencing, because 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://papilio.ab.a.u-tokyo.ac.jp/silkbase/>) for *Bombyx mori* or Flybase (<http://flybase.bio.indiana.edu/>) for *Drosophila* cell lines. Although 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>), Silkbase and Flybase are the best resources available at this time. Once the sequence is obtained, design can be completed relatively quickly at one of the aforementioned websites.

### 2.2. Making the DNA Template

1. RNA extraction kit. RNA extraction kits are widely available from a number of resources. This protocol makes use of the RNAqueous<sup>®</sup>-4PCR kit from Ambion (Houston, TX). This kit includes:
  - a. Lysis/binding buffer. This buffer contains  $\beta$ -mercaptoethanol, sodium orthovanadate, and a proprietary mixture intended to inhibit RNases, 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. DNase 1.
  - e. DNase inactivation reagent. This polymeric emulsion binds to the DNase 1 and removes it from solution.
  - f. Elution buffer. This buffer contains 0.1 mM EDTA.
2. Nuclease-free water. This is available from Invitrogen 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 Invitrogen. This III kit includes:
    - a. Deoxynucleotides (dATP, dCTP, dGTP, dTTP).
    - b. DTT (reducing agent).
    - c. MgCl<sub>2</sub>.
    - d. OligodT primers.
    - e. 10X Reaction buffer.
    - f. RNase inhibitor.
    - g. Superscript III RNA-dependent DNA polymerase.
    - h. RNase H and RNase 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<sub>2</sub> solution. This solution usually comes with the DNA polymerase to increase the MgCl<sub>2</sub> concentration when the PCR result is unsatisfactory.

### 2.3. *In Vitro dsRNA Synthesis*

1. T7 or SP6 RNA polymerase kit. There are several manufacturers of RNA polymerase kits. The following list of materials is from a MEGAscript™ kit sold by Ambion. This kit includes:
  - a. 80 μL Enzyme mix (T7).
  - b. 80 μL 10X 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 DNase 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 RNase H from previous steps.

### 2.4. Transfecting Sf-21 Cells in Culture

1. Sf900 II serum-free medium (SFM) (Gibco). This serum-free media has L-glutamine added.
2. Sf-9 cells (Invitrogen). 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) (Gibco).

## 3. Methods

### 3.1. Designing dsRNA

1. Determine which genes are of interest for silencing. The aforementioned websites relating information on *B. mori* (<http://papilio.ab.a.u-tokyo.ac.jp/silkbase/>) and *Drosophila* (<http://flybase.bio.indiana.edu/>) will likely provide the most information for both potential targets and nonspecific effects.
2. Design should make use of the websites listed in **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 next.
4. In deciding on how much dsRNA you will need, a rule of thumb is 30 µg per well in a six-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 DNase 1 for 30 min at 37°C (**step 1** of the optional DNase digest).

3. Stop the DNase 1 digest with DNase inactivation reagent (manufacturer's step 3 of optional DNase digestion). Spin down the DNase inactivation reagent at 10,000g for 1 min (step 4 of optional DNase digestion). Carefully transfer the RNA to a fresh 1.5-mL microfuge tube, being careful not to disrupt the DNase inactivation reagent pellet on the bottom of the tube.
4. Aliquot 190  $\mu$ L of nuclease-free water into a nuclease-free microcentrifuge tube. Measure the yield of RNA by carefully pipetting 10  $\mu$ L RNA into the 190  $\mu$ L nuclease-free water, mixing well, and measuring the absorbance at 260 and 280 nm (uvUV). 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/ $\mu$ L (RNA  $Abs_{260}$  of 1 = 40 ng/ $\mu$ L).
5. Use 50–200 ng of RNA per reaction with the Superscript III kit (Invitrogen) 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  $\mu$ L of reverse transcript from **step 5**, 2  $\mu$ L 10X reaction buffer, 1  $\mu$ L of each 10  $\mu$ M primer stock (not the T7 stock, that will be used later), 2  $\mu$ L dNTP mix, 9  $\mu$ L nuclease-free water, and 1  $\mu$ L 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** to make the final DNA template with the T7 primer sequence. This time use the primers with the T7 sequence added. Use 5  $\mu$ L of the PCR reaction from **step 6** instead of the reverse transcript. For making of this final template, 32 cycles or more 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  $\mu$ g dsRNA, two 20- $\mu$ L PCR reactions will usually suffice.
8. 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 as per manufacturer's instructions using the MEGAscript kit from Ambion (manufacturer's steps 1–5). To get  $> 500$   $\mu$ g of dsRNA, five reactions per dsRNA to be synthesized is recommended. Use  $2 \times 20$   $\mu$ L of final DNA template (with T7 sequence added) and no water in assembling the reactions. This will give 40  $\mu$ L of template + 40  $\mu$ L of rNTP + 10  $\mu$ L of 10X buffer + 10  $\mu$ L

of enzyme mix = 100  $\mu$ L reaction volume. The synthesis incubation should be extended to 5 or 6 h.

2. Extract the dsRNA using phenol:chloroform extraction as per MEGAscript kit instructions (manufacturer's "recovery of the RNA" step 4). The isopropanol precipitation should be carried out overnight at  $-20^{\circ}\text{C}$ . For increased purity, after overnight incubation in isopropanol, the pellet should be washed twice in  $-20^{\circ}\text{C}$  ethanol. Remove as much ethanol as possible from the pellet using a pipet. 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  $\mu$ L nuclease-free water at first and measure the Abs<sub>260</sub>. Adjust the concentration to 3.3  $\mu\text{g}/\mu\text{L}$  for best results when silencing.
4. Separate the dsRNA strands at  $65^{\circ}\text{C}$  for 30 min by floating the microcentrifuge tube containing dsRNA in a water bath. Transfer some  $65^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$ .

### 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 six-well plates and allow to grow overnight at  $27^{\circ}\text{C}$  in an incubator.
2. Make stock solutions of 15–30  $\mu\text{g}$  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 3 mL X number of wells in total experiment (treatments and controls) of Sf900 II SFM + 10% FBS.
4. Remove the 2 mL Sf900 II from each well of the six-well plate from **step 1** and replace it with 1 mL of the dsRNA containing solution. Swirl wells to mix. Place the six-well plates back into the incubator for 50 min.
5. Remove the 1 mL of dsRNA-containing media from the cells and replace with 3 mL of Sf900 II SFM + 10% FBS (from **step 3**).
6. Allow the cells to incubate for 36–72 h at  $27^{\circ}\text{C}$ .
7. Assay for gene expression (*see Note 2*).

## 4. Notes

1. In vivo expression of dsRNA in Sf-21 cells has not yet been reported. However, in vivo silencing for *Drosophila* S2 cell culture has been reported (9). 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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## Using the Baculovirus/Insect Cell System to Study Apoptosis

Nor Chejanovsky

### Summary

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. Because of 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 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, which 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). Because of 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). 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 (4,5). 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

From: *Methods in Molecular Biology*, vol. 338: *Baculovirus and Insect Cell Expression Protocols*, 2/e  
Edited by: D. W. Murhammer © Humana Press Inc., Totowa, NJ

the viral replication cycle and polyhedra production (6). This fact and the high conservation of the apoptotic machinery across the animal kingdom can be implemented to isolate genes that suppress apoptosis from vertebrates and invertebrates (7–10). 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 because 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 23]). 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*) or are in the process of it (Lepidopteran genome project).

## 2. Materials

### 2.1. Chemicals and Solutions

1. Restriction enzyme *DpnI* (Fermentas GMBH, Germany).
2. Ethanol absolute analytical grade (Bio-Lab Ltd., Jerusalem, Israel) diluted to 95% (v/v) with sterile DDW.
3. pIEx-1 plasmid (Novagen, Darmstadt, Germany).
4. pBlueP49 stop plasmid (11).
5. Phenol:chloroform-IAA (isoamyl alcohol): a mixture of 25:24:1 parts of phenol, chloroform, and isoamyl alcohol respectively (Bio-Lab Ltd.).
6. Sodium acetate anhydrous (Bio-Lab Ltd.) diluted in sterile DDW to a 3M solution.
7. Sodium chloride molecular biology grade (Sigma, St. Louis, MO).
8. Tfx™-20 (Promega, Southampton, UK).
9. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
10. TNM-FH serum-free medium (Sigma).
11. Complete TNM-FH medium: TNM-FH serum-free medium (Sigma), 10% heat-inactivated fetal bovine serum (Sigma), antibiotics (penicillin 50 U/mL and streptomycin 50 µg/mL, Sigma).
12. Extraction buffer: 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% SDS supplemented with 0.1 mg/mL proteinase K (Sigma).
13. Oligonucleosome extraction buffer: 10 mM Tris (pH 8.0), 1 mM EDTA, 1% sodium dodecyl sulfate (SDS) buffer containing 70 µg of proteinase K per milliliter.

14. PCR reaction mixture: 25 ng DNA, 2  $\mu$ L 10X reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8% [w/v] Nonidet-P40), 0.4  $\mu$ L of each dNTP (10 mM, Promega), 1  $\mu$ L of each primer (50 ng/ $\mu$ L), 0.2  $\mu$ L Pfu-Taq Polymerase (1 U, Stratagene, Amsterdam, The Netherlands), ddH<sub>2</sub>O to a final volume of 20  $\mu$ L.

## 2.2. Cells and Viruses

1. *S. frugiperda* Sf-9 and *Trichoplusia ni* Tn-368 cells (kindly donated by Dr. Max D. Summers, Texas A&M University, and by Dr. Just M. Vlak, Wageningen University, The Netherlands, respectively) were maintained and propagated in complete TNM-FH medium (Sigma).
2. Wild-type (wt) AcMNPV E-2 strain, *Spodoptera littoralis* nucleopolyhedrovirus SINPV E-15 strain and  $\nu\Delta$ P35K/pol+, a mutant of AcMNPV lacking the *p35* gene (9).
3.  $\nu\Delta$ P35K/pol+ genomic DNA, i.e., DNA extracted from  $\nu\Delta$ P35K/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, resulting in formation of polyhedra in the cell nuclei that are easily detected using a light microscope (Fig. 1A). In contrast, transfecting cells with  $\nu\Delta$ P35K/pol+ genomic DNA results in extensive apoptosis (Fig. 1B). Thus, the ability of a gene to suppress apoptosis can be assayed by cotransfecting Sf-9 cells with DNA (plasmid, cosmid, and so on) containing the gene of interest and  $\nu\Delta$ P35K/pol+ genomic DNA. Suppression of apoptosis rescues  $\nu\Delta$ P35K/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 SINPV genome (9).

#### 3.1.1. Infection of Insect Cells With Baculoviruses

1. Seed Sf-9 or Tn-368 in a 12-well plate ( $5 \times 10^5$  cells/well) and allow to attach at least for 4 h at 27°C.
2. Replace the medium with the infecting viral inoculum (200  $\mu$ L, of the desired viral multiplicity of infection [MOI] diluted in TNM-FH serum-free medium), added drop wise with gently rocking the plate (see Note 1).
3. After 1 h of adsorption the medium is replaced by complete TNM-FH medium.

#### 3.1.2. Isolation of AcMNPV DNA

Wild-type or mutant (*p35*-null) AcMNPV DNA are isolated from Sf-9 or Tn-368 cells, respectively, infected with the corresponding viruses at 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.

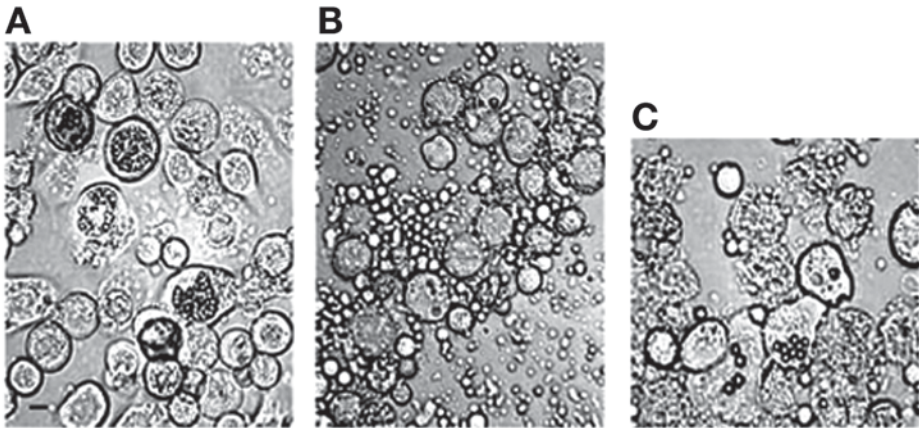


Fig. 1. (A,B) infection of Sf-9 cells with *Autographa californica* multiple nucleopolyhedrovirus and v $\Delta$ P35K/pol+ showing polyhedra-formation and apoptosis, respectively. (C) Sf-9 cells transfected with v $\Delta$ P35K/pol+ and pAN (expressing *p49*) rescuing viral replication and showing polyhedra. Bar = 2  $\mu$ m.

2. Dilute the viral inoculum (1 mL) in TNM-FH serum-free medium and add drop wise with gently rotating the plate.
3. After 1 h of adsorption replace the medium with fresh TNM-FH complete medium and incubate the cells at 27°C for 4–5 d.
4. Transfer the cell supernatants to 15-mL conical tubes and pellet the cells by centrifugation at 1000g for 10 min at 4°C.
5. Centrifuge the resulting cell supernatants (from **step 4**) at 12,000g for 1 h at 4°C to pellet the budded virus (*see Note 3*).
6. Resuspend the cell pellet in 350  $\mu$ L extraction buffer and incubate at 37°C for 4 h.
7. Extract the DNA samples with equal volumes of phenol:chloroform-IAA (isoamyl alcohol), mix by turning it the tube up and down to avoid braking of the DNA.
8. Centrifuge at 12,000g for 1 min and transfer the aqueous upper phase to a new sterile Eppendorf tube.
9. To the aqueous phase (from **step 8**) add 3 M sodium acetate (1/10 of the volume) and cold (–20°C) 95% ethanol (two volumes). Incubate at –20°C for 15 min.
10. Centrifuge at 12,000g for 10 min.
11. Wash the pellet with cold (–20°C) 70% ethanol and air-dry briefly.
12. Resuspend in 100  $\mu$ L TE buffer.
13. Quantify the amount of DNA by measuring the OD<sub>260</sub> of the solution (*see Note 4*).

### 3.1.3. Transfection Mixture Preparation

1. Solution A: to a polystyrene sterile tube add 200  $\mu$ L TNM-FH serum-free medium. Add 3  $\mu$ L Tfx-20.

2. Solution B: in a sterile Eppendorf tube add 200  $\mu$ L TNM-FH serum-free medium. Add 1  $\mu$ g of the relevant DNA (e.g., 500 ng of tested DNA and 500 ng  $\nu\Delta P35K/pol+$  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 in a 12-well plate Sf-9 cells ( $5 \times 10^5$  cells per well) 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 drop wise with gently rocking of 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 3.1.5.1.** and **3.1.7.**).

#### 3.1.5. Induction of Apoptosis in Insect Cells

*S. frugiperda* cells can be induced to apoptosis by various stimuli (e.g., baculovirus infection, ultraviolet irradiation, and so on). We utilized two approaches to induce apoptosis of Sf-9 cells:

1. Infection with AcMNPV null mutant of *p35* (e.g.  $\nu\Delta P35K/pol+$ ).
2. Transfection with the genomic DNA of a *p35* mutant.

##### 3.1.5.1. INDUCING APOPTOSIS BY BACULOVIRUS INFECTION

1. Infect Sf-9 cells with  $\nu\Delta P35K/pol+$  budded virus isolated in **Subheading 3.1.2., step 5** (MOI 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 postinfection (pi) and increases with time (**Fig. 1**).

##### 3.1.5.2. INDUCING APOPTOSIS BY TRANSFECTION

1. Plate Sf-9 cells  $4 \times 10^5$ /well in a 12-well plate.
2. The next day remove the medium and add the transfecting mixture prepared in **Subheading 3.1.3.** containing only  $\nu\Delta P35K/pol+$  genomic DNA transfecting mixture rotating the plate to avoid increase of the local concentration of the transfected 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.

#### 3.1.6. Monitoring Apoptosis

Apoptosis of Sf-9 cells can be followed as described next.

### 3.1.6.1. DIRECT MICROSCOPIC OBSERVATION OF THE BLEBBING CELLS

1. Cell blebbing as a result of 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**).

### 3.1.6.2. EXTRACTION OF FRAGMENTED DNA (OLIGONUCLEOSOMES)

1. Extract DNA oligonucleosomes for 2 h at 37°C from virus-infected Sf-9 cells with 400  $\mu$ L of “oligonucleosome extraction buffer.”
2. Add 5 M NaCl (to a final concentration of 1 M) (i.e., 100  $\mu$ L added to the 400  $\mu$ L from **step 1**) and incubate the extracts overnight at 4°C.
3. Treat the extracts with 500: 500- $\mu$ L phenol-chloroform-IAA (i.e., add to the 500  $\mu$ L from **step 2**).
4. Separate the aqueous phase by centrifugation (12,000g 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 12,000g for 15 min.
7. Rinse the pellet with 70% cold ethanol (–20°C) and centrifuge again.
8. Resuspend the DNA in 60  $\mu$ L TE and analyze it by standard agarose electrophoresis using a 1% gel (**Fig. 2**).

### 3.1.7. Genetic Screen and Rescue of AcMNPV Replication

1. One microgram of  $\nu\Delta$ P35K/pol+ genomic DNA and 1  $\mu$ g of test DNA (e.g., SINPV cosmids) are cotransfected into  $4 \times 10^5$  Sf-9 cells as described in **Subheading 3.1.4**. (see **Note 8**).
2. Examine the cells 3–4 d 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**).

### 3.1.8. Identification of the Apoptosis-Suppressing Gene

This is achieved by subcloning the tested DNA (if necessary).

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 indeed 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**).

### 3.1.9. Isolation of Recombinant Baculoviruses

Recombinant baculoviruses bearing the new apoptosis suppressor gene can be isolated because 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  $\nu\Delta$ P35K/pol+ genomic DNA (**Fig. 3**) as described in **Subheading 3.1.7**.

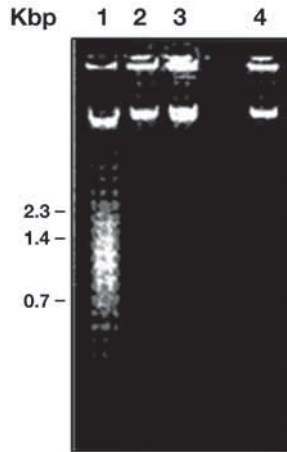


Fig. 2. Oligonucleosomes extracted from Sf-9 cells infected with  $v\Delta P35K/pol+$  (lane 1). Extracts from Sf-9 cells infected with *Autographa californica* multiple nucleopolyhedrovirus (lane 2) and a recombinant  $v P35K/pol+$  that bears the *p49* gene (lane 3). Mock infected cells (lane 4). Molecular markers are indicated on the left. (Reproduced from ref. 9 with permission of ASM journals.)

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 pipet or a micropipet and place it in an Eppendorf tube containing 1 mL complete TNM-FH medium and vortex.
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 *iel* promoter available in the pIEx-1 plasmid or the Hsp70 promoter of *Drosophila* (10) and performing the marker-rescue assay described in **Subheading 3.1.7.**

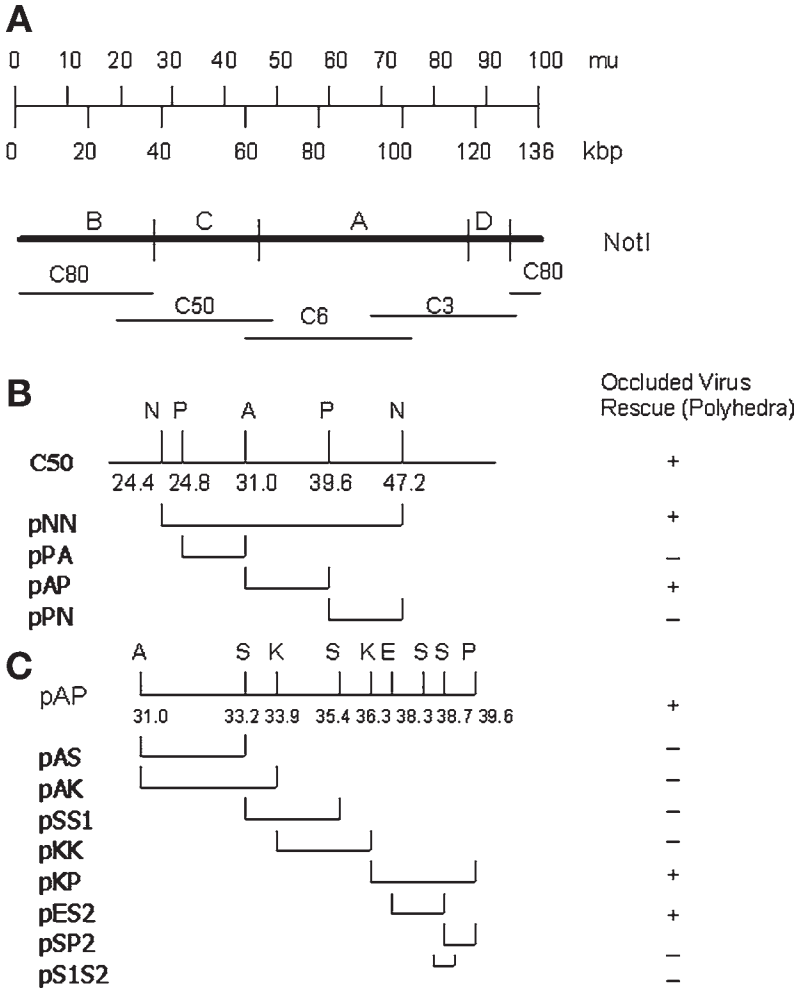


Fig. 3. Rescue of *Autographa californica* multiple nucleopolyhedrovirus occluded viruses by cosmids and plasmids bearing SINPV DNA fragments. (A) *NotI* linear restriction map of SINPV. The scales above indicate SINPV map units (m.u.) and kilobase pairs (kbp). The bars below (C80, C50, C3, and C50) represent the various overlapping cosmids of the genomic SINPV 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 v 35K/pol+, as detected by the presence of polyhedra in the nuclei of Sf-9 cells cotransfected with v 35K/pol+ and plasmid DNA. N, *NotI*; P, *PstI*; A, *apaI*. (C) Restriction map of the *Apal-PstI* region corresponding to SINPV 31.0 to 39.6 m.u. S, *Sall*; K, *KpnI*; E, *EcoRI*. Also, subclones able to rescue vΔ35K/pol+ polyhedra formation as in B are indicated. (Reproduced from ref. 9 with permission of ASM journals.)

### 3.3. Evaluating Structural Motifs of Apoptosis-Suppressing Proteins

The transfection rescue approach described in **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 (3D) structure of P49 utilizing Swiss-Model (<http://swissmodel.expasy.org/SWISS-MODEL.html>) and 3D-PSSM Web server Biomolecular Modeling Laboratory at The Imperial Cancer Research Fund (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>) and introduced site directed mutations in P49 using the overlap extension PCR with complementary primers containing the desired mutation in the plasmid pBlue-49-stop (**II**) (see **Fig. 4** and **Table 1**). The method is described next.

#### 3.3.1. Site-Directed Mutagenesis Using Overlap Extension PCR

1. Template: plasmid DNA bearing the identified gene (e.g., pBlue- P49 stop).
2. Primers 25- to 30-mers, one pair of complementary primers of equal length for each mutant with six over-hanging noncomplementary bases at the 3'-terminus of each primer to enable better stability at the transformation step (see **Note 8**).
3. Reaction conditions: 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. 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 *Escherichia 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.

#### 3.3.2. Quantitative Evaluation of the Marker Rescue Assay

1. Transfect each plasmid containing the desired mutation with  $\nu\Delta P35K/pol+$  genomic DNA into Sf-9 cells as described in **Subheading 3.1.7**.
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 nonapoptotic polyhedra-positive plaque forming units are considered functional, whereas mutations that yield less than 8000 plaques are considered nonfunctional (see **Notes 10** and **11**).

## 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.

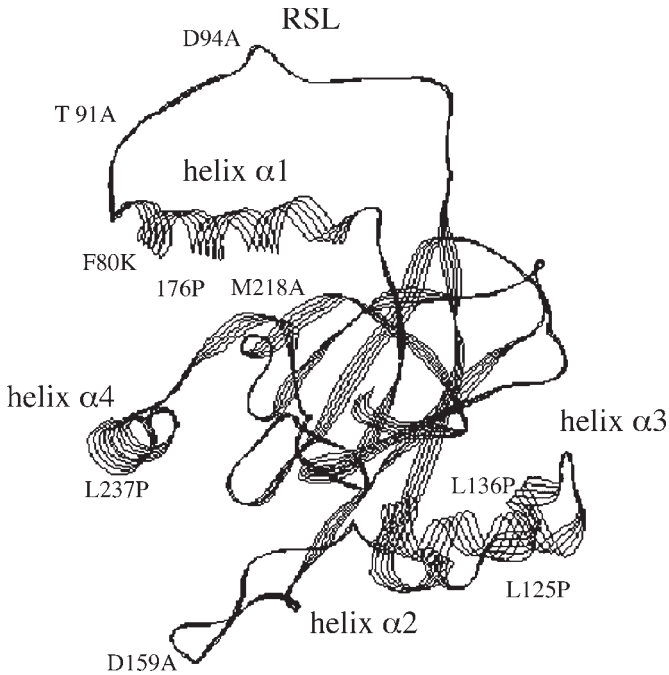


Fig. 4. Partial three-dimensional structure of P49 (amino acid residues 1–299), showing predicted several domains important for its function: (A)  $\beta$ -core, composed of a  $\beta$ -barrel domain with a large insertion which forms the reactive site loop (RSL). The RSL begins at the amphipatic  $\alpha 1$  helix (between Val 69 to Phe 83) and traverses the  $\beta$ -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  $\beta$ -barrel; (B) three additional  $\Delta\alpha$  helical domains,  $\alpha 2$  (between Gly 115 to Asn 127),  $\alpha 3$  (between Tyr 133 to Pro 146), and  $\alpha 4$  (between Ile 231 to 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. 11](#).)

2. Tn-368 cells do not undergo apoptosis by infection with  $v\Delta P35K/pol+$  budded virus and are suitable to isolate genomic DNA of this virus.
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  $\mu$ L Tfx-20 per 1  $\mu$ g 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.

**Table 1**  
**Structural Domains Important for P49 Function P4**

9 predicted domain	Mutation	Functional effect <sup>a</sup> (Apoptosis suppression)
helix $\alpha$ 1	L76P	–
helix $\alpha$ 1	F80K	–
helix $\alpha$ 2	L125P	–
helix $\alpha$ 3	L136P	+
helix $\alpha$ 4	L237P	–
$\beta$ -sheet	M218A	–
Turn	D159A	+
Reactive site loop (RSL)	T91A	–
RSL	D94A	–

<sup>a</sup>The ability of the various P49 mutants to rescue the replication of  $\nu\Delta P35K/pol+$  was determined by cotransfecting them with genomic  $\nu\Delta P35K/pol+$  DNA to Sf-9 cells. Rescuing mutants (+) yielded more than 60,000 nonapoptotic polyhedra-positive plaque-forming units per milliliter, whereas nonrescuing mutants (–) yielded less than 8000 plaque-forming units per milliliter. The helical motifs  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 4, the residue M218 and the residues T91 and D94 of the RSL are necessary for the antiapoptotic function of P49 (adapted from **ref. 11**).

7. It is very important that the transfection mixture should be prepared gently following the given directions. Otherwise, some cell lysis and blebbing can sometimes be observed owing 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'-CGACCGTGACCGCTGGCGGTGGAGCCGAT-3' and 5'-CTCCACCGCCAGCGGTCACGGTCGCCGAT-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 because some mutants may show low performance owing to partial loss of function.

## Acknowledgments

The author would like to thank Hadassah Rivkin for her excellent technical support. This work was supported by Israel Science Foundation Grant 426/99-3 and in part by Fogarty International Research Collaboration Award Grant TW01219.

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## Generation of Envelope-Modified Baculoviruses for Gene Delivery Into Mammalian Cells

Christian Hofmann

### Summary

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 will give clues as to 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

Ten years ago, we genetically engineered baculoviruses that can efficiently deliver and express genes into mammalian cells (1). The only prerequisite for the expression of a gene transferred by baculovirus was 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 owing to their “nonmammalian-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 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 will be adequately coexpressed in insect cells even from 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 without signal sequence and glycosyl-phosphatidylinositol-anchor 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).

#### *1.2.2. Targeted Vectors*

As described in **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 epidermal growth factor-receptor positive cells in comparison with vectors having the wild-type envelope.

### 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. 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 or *Kpn*I or *Pst*I and *Eco*RV, Klenow enzyme, T4 DNA ligase, and so on).
4. Luria-Bertani medium.
5. Agar plates.
6. Selection agent (ampicillin).
7. Competent *Escherichia coli* cells DH5- $\alpha$  (Invitrogen, Carlsbad, CA).
8. Kits 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.).
10. Phenol/chloroform/isoamylalcohol isoamylalcohol solution (25:24:1) (Carl Roth GmbH, Karlsruhe, Germany).

### 2.2. Insect Cell Culture

1. Sf-9 cells (PharMingen, EMD Bioscience).
2. EX-CELL™ 401 medium (JHR Bioscience, Hampshire, UK; 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 U/L penicillin.
4. Tissue culture plates and flasks (Falcon).
5. Cellspin-platform and spinner flasks (Integra Bioscience, Fernwald, Germany; distributed in the United States 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).
3. Recombinant transfer plasmid from **Subheading 3.1.** (pBacsurf\_X\_mam).
4. Falcon polystyrene tubes (Becton-Dickinson, Heidelberg, Germany).
5. Lipofectin (Gibco, Karlsruhe, Germany; Invitrogen).
6. Sterile distilled water.

## 2.4. Titration by Plaque Assay

1. Insect cell culture material (*see Subheading 2.2.*).
2. Seaplaque-Agarose™ (FMC, Rockland, MD).
3. Sterile distilled water.
4. 60-mm Plates (Falcon).
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 (**Subheading 3.3.2., step 15**).
3. 1-L Spinner flasks (Cellspin, Integra, Fernwald, Germany; distributed in the United States by Argos Technologies Inc.).

## 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<sub>2</sub>HPO<sub>4</sub>, 10.5 mM KH<sub>2</sub>PO<sub>4</sub>, 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 96,000g, e.g., SW28 and SW 40 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% sodium dodecyl sulfate (SDS), 300 mM 2-mercaptoethanol, 0.0025% bromophenol blue.
3. SDS polyacrylamid gels (8%).
4. 1 mM Dithiothreitol.
5. Rainbow molecular weight protein marker (Amersham [now part of GE Health Care], 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, 192 mM glycine, 5% (v/v) methanol.
9. Hybond-C extra membranes (Amersham Pharmacia Biotech, Freiburg, Germany; Amersham [now part of GE Health Care]).

10. Blocking buffer: 5% (w/v) milk powder, 0.1% (v/v) Tween-20 in PBS.
11. Primary antibody against AcV5 (9) 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 [now part of GE Health Care]).

### 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.
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 *EcoRV*-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 *EcoRV*-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 *SmaI* or *KpnI* or *PstI* into pBACsurf\_mam to generate pBACsurf\_X\_mam (see Note 2).

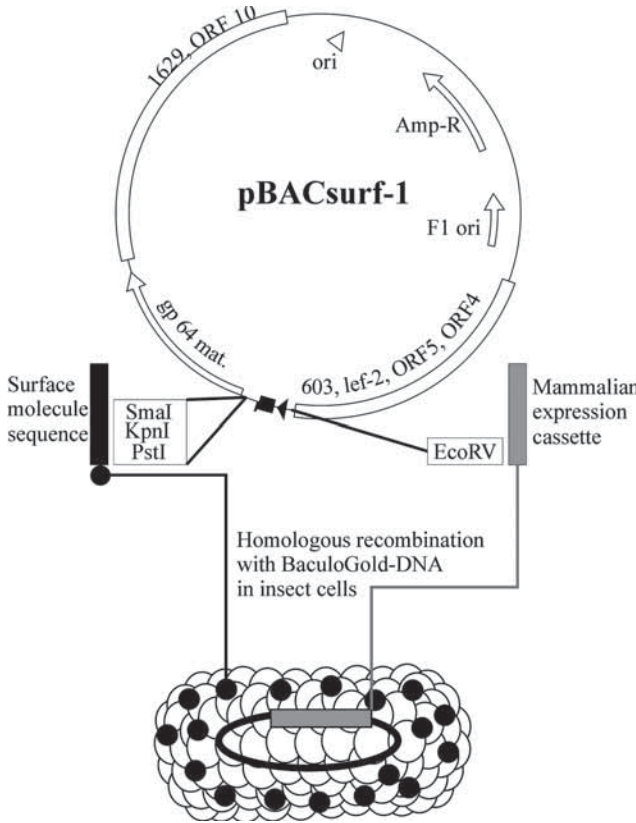


Fig. 1. Map of baculovirus transfer plasmid pBACsurf-1 with insertion sites for envelope modification and expression of genes in mammalian cells. Viruses are generated by homologous recombination with the transfer vector and genomic baculovirus DNA in insect cells. Black arrow: polyhedrin promoter; black 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. *SmaI*, *KpnI*, and *PstI* are restriction sites to insert surface modification sequences; *EcoRV* can be used to insert a mammalian expression cassette.

#### 3.1.4. Baculovirus Genomic DNA

BaculoGold is a modified *Autographa californica* multiple nucleopolyhedrovirus baculovirus DNA that contains a lethal deletion and does not code for viable virus. Cotransfection 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**).

## 3.2. Insect Cell Culture

### 3.2.1. Serum-Free vs 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 (**Subheading 3.3.3., step 17**). The use of serum-free medium facilitates purification because of the absence of FCS, but results—at least in our hands—is 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<sup>2</sup> tissue culture flasks with  $2 \times 10^6$  Sf-9 cells (50–70% confluent). One flask will be the experimental cotransfection flask, the other a negative control flask.
2. After cell attachment, wash cells gently three times with 3 mL EX-CELL 401 medium. Finally, add 1 mL EX-CELL 401 medium.
3. Experimental cotransfection: combine 0.5  $\mu$ g BaculoGold DNA and 3  $\mu$ g recombinant Baculovirus Transfer Vector in a polystyrol Polystyrol tube, add sterile water to a total of 50  $\mu$ L, and mix by pipetting up and down (*see Note 3*).
4. Mix 20  $\mu$ L lipofectin and 30  $\mu$ L sterile water in a separate polystyrol Polystyrol tube.
5. Add DNA solution (from **step 2**) drop-by-drop to Lipofectin/water mixture (from **step 3**).
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 cotransfection 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 d.
10. After 5 d, cells of the experimental cotransfection 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 cotransfection 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). 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  $10^{-82}$ ) of virus-containing supernatant (e.g., **Subheading 3.3.1., step 12**) 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 monolayer 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 d 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 virus from a “high” dilution plate by picking with a pipet into the plaque and transferring virus in agarose into an Eppendorf tube prefilled with 1 mL complete EX-CELL-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, and so on).
14. Freeze one (back-up) aliquot (500  $\mu$ L) at  $-80^{\circ}\text{C}$  (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. Seed  $2 \times 10^6$  Sf-9 cells (50–70% confluent) into 25-cm<sup>2</sup> tissue culture flasks. Allow them to attach for 15 min and change to 4.5 mL fresh complete EX-CELL 401 medium.
2. Add the 500  $\mu$ L of your low titer A0 recombinant stock (**Subheading 3.3.1., step 13**) to the flask.
3. Incubate the cells at 27°C for 5 d. Check for signs of infection 3 d postinfection (pi) (floating cells in comparison to a confluent monolayer in the control flask).
4. Harvest the supernatant from the flask and centrifuge at 500g 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  $\mu$ L at  $-80^{\circ}\text{C}$  as A1-back-up (see **Note 4**).

6. At this stage, rather than determining the virus titer, which is normally around  $10^6$ – $10^7$  per milliliter, try to amplify your specific inserts by PCR using 1  $\mu$ L (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<sup>2</sup> 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 d 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 (500g, 15 min).
10. Store the virus supernatant (A2) in a sterile tube at 4°C in the dark and freeze 500  $\mu$ L at –80°C as A2-back-up (*see Note 4*).
11. Proceed with amplification (**steps 7–10**) by using three 225-cm<sup>2</sup> 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 (**Subheading 3.3.2., steps 1–11**). The titer should reach about  $1$ – $2 \times 10^8$ /mL at this stage.
13. Amplification in spinner flasks: seed approx  $5 \times 10^5$  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 approx 24 h (*see Chapter 1*).
14. Cultivate cells (*see Subheading 3.2.1.*) for about 2–3 d 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 multiplicity of infection (MOI) of one. Repetitive infections with an MOI of substantially higher than one will select for deletion mutants that may no longer bear your inserts.
15. Cultivate cells for an additional 1.5–2.5 d (absolute maximum). 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 500g for 15 min and then centrifuge the resulting supernatant at 5000g for 15 min. Virus containing insect cell medium should be clear (*see Note 5*).
17. Titrate the A4 by plaque assay (**Subheading 3.3.2., steps 1–11**) 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 80,000g 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 in **Subheading 3.3.5**.

### 3.3.5. Virus Purification (see **Note 6**)

1. For 2 mL concentrated virus (**Subheading 3.3.4., step 5**), 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) (**Subheading 3.3.4., step 5**) 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 10-fold.
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 volume covering both, infectious and noninfectious viruses. By dividing this number by the number of infectious viruses from plaque assay experiments, you end up with 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 your specific construct (see **Subheading 1.2.**) and should be performed with A4p preparations (**Subheading 3.3.5., step 8**). For example, we generated complement-resistant vectors by incorporating decay acceleration factor into the viral envelope. In addition, the vectors were able to mediate  $\beta$ -galactosidase expression in insect and mammalian cells. The functionality was therefore tested in a Sf-9 insect cell-based and a 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 nonsusceptible cell lines. Vaccine vectors can be tested based on their ability to mediate a humoral or cellular immunity against displayed or expressed antigens after injection into animals.

### 3.4.2. Immunoblotting

To determine the composition of envelope modification, vectors from A4p preparations (**Subheading 3.3.5., step 8**) with wild-type envelope and modified envelope should be analyzed by immunoblotting. If analysis is done under nonreducing and reducing conditions and development is with a specific antibody against the displayed protein and gp64, then four identical gels/blots are required. Nonreducing conditions allow for detection of multimeric forms of your 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 of 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 sure that equal amounts of virus protein is loaded onto the gel.
2. Load onto SDS polyacrylamid gels (8%) under nonreducing or reducing conditions (sample buffer + 1 mM dithiothreitol) 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<sup>2</sup>.
5. Block membrane for 90 min with blocking buffer.
6. Incubate membrane with primary antibody, e.g., AcV5 against gp64 in an 1:1000 dilution 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 five times with PBS and visualize bands by the ECL detection system.

### 3.4.3. Electron Microscopy

1. Prepare serial dilutions of 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 immuno-fluorescence studies.
4. Wash grids three 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 five times with PBS.
6. Stain preparations with phosphotungstic acid 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 (**1,10**). Gene delivery and expression in mammalian

cells depends on the promoter used, the not-yet known receptor on the surface and differences (**11**) 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<sup>2</sup> (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  $\mu$ L/cm<sup>2</sup>. 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 will not be discussed with a specific example. To experience this system, reporter gene expression cassettes, e.g.,  $\beta$ -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 21*).

## 4. Notes

1. This plasmid (pBacsurf\_mam) is the basis of your reference baculovirus vector with wild-type envelope. Always generate this virus according to procedure given in **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 five to save material and money. If you decide to scale down transfection, then use 24-well plates for cell culture and 96-well polystyrol Polystyrol plates with round bottom for the Plasmid/BaculoGold-Lipofectin mixture.
4. At these steps, we recommend to prepare back-ups, 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  $\mu$ M to the cells prior to the transduction procedure derepresses transgene expression and leads in most cells to increased infection efficiencies—the compounds are, however, to a certain extent toxic the cells.

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