Chapter 16

Transforming Lepidopteran Insect Cells for Continuous Recombinant Protein Expression

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Abstract

The baculovirus expression vector system (BEVS) is widely used to produce large quantities of recombinant proteins. However, the yields of extracellular and membrane-bound proteins obtained with this system are often very low, possibly due to the adverse effects of baculovirus infection on the host insect cell secretory pathway. An alternative approach to producing poorly expressed proteins is to transform lepidopteran insect cells with the gene of interest under the control of promoters that are constitutively active in uninfected cells, thereby making cell lines that continuously express recombinant protein. This chapter provides an overview of the methods and considerations for making stably transformed lepidopteran insect cells. Techniques for the insertion of genes into continuous expression vectors, transfection of cells, and the selection and isolation of stably transformed Sf-9 clones by either colony formation or end-point dilution are described in detail.

Key words Insect cells, Baculovirus, Baculovirus expression vector system, BEVS, Cell transformation, Genetic engineering

1 Introduction

1.1 Rationale

Protein production with the BEVS usually involves using a recombinant baculovirus in which the polyhedrin open reading frame has been replaced with an open reading frame encoding the protein of interest [1–4]. Because the polyhedrin protein is not required for infection and replication, recombinant baculoviruses can be used to infect cultured insect cells or larvae. During infection, the polyhedrin promoter will induce the production of a very large quantity of mRNA derived from the gene of interest, which can be translated to produce large amounts of the corresponding protein. The actual yield obtained with this system can vary significantly from protein to protein, but is often measured in the 100s of mg/L of baculovirus-infected cell cultures. In addition, baculovirus-infected insect cells and larvae can perform most of the co- and post-translational protein modifications that occur in mammalian

cells, such as proteolytic processing, *N*- and *O*-glycosylation, phosphorylation, acylation, N-terminal acetylation, C-terminal methylation, and α-amidation (*see* Chapter 18). The capacity of the BEVS to produce large quantities of recombinant proteins bearing eukaryotic modifications is among its chief advantages.

A major disadvantage of the BEVS is that the yields of most membrane-bound and secreted proteins, which include many important gene products, are significantly lower (1–20 mg/L) than the yields obtained with intracellular proteins. There is some evidence to suggest that the relatively lower yields of secretory pathway proteins might reflect adverse effects of baculovirus infection on host secretory pathway function [5]. This observation led to the development of a new approach to recombinant protein production, which involved stable transformation of lepidopteran insect cell lines with plasmids encoding the protein of interest under the control of a constitutively active promoter [6]. The promoter initially used to develop this approach was derived from a baculovirus immediate early gene, *ie-1* [7, 8]. Plasmids encoding foreign proteins under *ie-1* control can integrate into the cellular genome and, because the *ie-1* promoter is active in uninfected lepidopteran insect cells, this approach circumvents the need to infect the cells with a recombinant baculovirus.

The first study utilizing this approach showed that transformed Sf-9 cell lines constitutively expressing *E. coli* β-galactosidase under the control of the *ie-1* promoter produced far less β-galactosidase than Sf-9 cells infected with a conventional baculovirus expression vector. This was expected, as the *ie-1* promoter is significantly weaker than the polyhedrin (*polh*) promoter and β-galactosidase, a cytoplasmic protein, can be produced at very high levels by the conventional baculovirus-insect cell system. However, this study also showed that transformed Sf-9 cells constitutively expressing human tissue plasminogen activator (t-PA), a secretory pathway protein, produced about as much extracellular product under *ie-1* control as the Sf-9 cells infected with a conventional baculovirus vector [6]. While the baculovirus-infected cells produced more total (intracellular and extracellular) t-PA, most of the t-PA produced by this conventional system was not secreted. Hence, the efficiency of t-PA secretion was considerably higher with the uninfected, transformed cell lines than with the baculovirus-infected cells, supporting the idea that baculovirus infection might, indeed, have an adverse effect on host secretory pathway function.

The aforementioned study was the first to demonstrate that stably transformed lepidopteran insect cell lines could be used to constitutively produce a foreign secretory pathway protein at a level comparable to that obtained with the conventional BEVS. Since then, Sf-9 cells have been transformed to constitutively express many other foreign genes under *ie-1* control [9]. The results of these subsequent studies confirmed the trends originally observed

by Jarvis and coworkers and revealed other advantages of using uninfected, transformed lepidopteran insect cell lines to express certain types of gene products (*see* **Note 1**). In addition, the original work has been reproduced and extended by the development of new expression constructs [10–20], the use of other selection markers [11, 12, 21], and the use of other lepidopteran insect cell lines [12, 14, 15, 22]. High-density culture systems for larger-scale expression of recombinant protein from stably transformed lepidopteran insect cell lines also have been evaluated [23, 24].

1.2 Basic Approach   The first step in producing a stably transformed lepidopteran insect cell line designed to continuously express a foreign protein is to insert a DNA sequence encoding the foreign protein of interest into a plasmid vector containing a promoter that is active in the parental cell line. The *ie-1* and *ie-2* promoters, the latter derived from another baculovirus immediate early gene [25], have been widely used for this purpose [6, 10, 11, 13], as have host promoters derived from a *B. mori* (silkworm) actin gene [14] and a *Drosophila melanogaster* (fruit fly) heat shock protein gene (*hsp70*) [12]. Baculovirus-derived enhancer elements, known as homologous DNA regions (*hr*s), have been used to stimulate *ie-1*-mediated transcription in stably transformed insect cells [10]. Interestingly, it has been shown that a baculovirus-derived *hr* element also can stimulate *B. mori* actin promoter-mediated transcription in transiently transfected insect cells, suggesting that this element might be able to augment actin-mediated expression in transformed cells, as well [26]. While this chapter focuses on the use of vectors for constitutive expression, vectors that provide for inducible expression in lepidopteran insect cells have also been developed and tested [16, 18–20, 27].

Plasmids encoding the gene of interest under the control of an appropriate promoter are introduced into lepidopteran insect cells by conventional DNA transfection procedures. In addition, one must introduce a constitutively expressible antibiotic resistance marker to enable selection of transformed cells that have incorporated functional copies of the gene(s) of interest into their genomic DNA. The antibiotic resistance marker may be placed either on the same plasmid as the gene of interest [13] or on a separate plasmid [6]. As for genes encoding proteins of interest, the antibiotic resistance markers are typically expressed under the control of baculovirus immediate early or insect cell promoters. A wide variety of resistance markers have been used, including neomycin (G418) [6], hygromycin B [21], puromycin [12], Zeocin™ [11], and blasticidin S [28]. However, it is important to recognize that lepidopteran insect cells maintained in the presence of the antibiotics routinely used for cell culture can be resistant to some of the antibiotics listed above [29].

Transformation vectors of the sort described in this chapter integrate into the chromosomal DNA of insect cells by illegitimate recombination. However, we should note that lepidopteran insect cells also have been transformed with *piggyBac* transposon-based vectors [15, 17, 27, 30, 31]. In the latter type of vector, genes encoding the recombinant protein of interest and a selectable marker are cloned between the inverted terminal repeats of a *piggyBac* transposon. This construct is then transfected into insect cells together with a helper plasmid encoding the *piggyBac* transposase. The transposase expressed by the helper plasmid catalyzes the transposition and precise integration of the *piggyBac* transposon carrying the gene of interest and marker gene into a tetranucleotide (TTAA) target site in host cell DNA. Cells transformed by these *piggyBac*-based vectors can be selected for antibiotic resistance using the same procedures as are used with standard transformation vectors [17, 27, 31]. An alternative (or supplement) to selection for drug resistance involves identifying and sorting transformed cells by fluorescence. If the vector used for cell transformation carries an expression cassette for a fluorescent protein such as green fluorescent protein (GFP), or if the coding sequence of the gene of interest is fused to a fluorescent protein coding sequence, then one can use fluorescence-activated cell sorting (FACS) with a flow cytometer to enrich for transformed cell subpopulations [15, 32].

Once selected, individual cells can be isolated and expanded to obtain transformed clones. Two methods that we have used to obtain clonal transformed insect cell derivatives are detailed below and outlined in Fig. 1. It is important to isolate multiple transformed clones and compare their abilities to produce the protein of interest, because antibiotic resistance does not guarantee expression of the unselected marker(s) and individual clones expressing the gene of interest typically exhibit a wide range of expression levels [6, 14]. Finally, once transformed clones expressing the gene of interest have been identified, it is important to recognize that this is not guaranteed to be a stable genetic trait. In order to validate the claim that one has isolated a "stably" transformed clone, it is necessary to monitor expression of the unselected marker routinely over a large number of passages. In our experience, transformed lepidopteran insect cells can express newly acquired transgenes over hundreds of passages. Furthermore, while the expression level is not necessarily constant from passage to passage, it should not steadily decline with increasing passage number if the transformed clone is a genetically stable cell line. In a recent study, we directly examined the impact of the presumed metabolic load imposed by the expression of multiple transgenes and found that the expression of six separate transgenes had no major impact on the growth properties or phenotypic stabilities of a transformed Sf-9 cell line, even after 300 passages (1.5 years) in continuous culture [27].
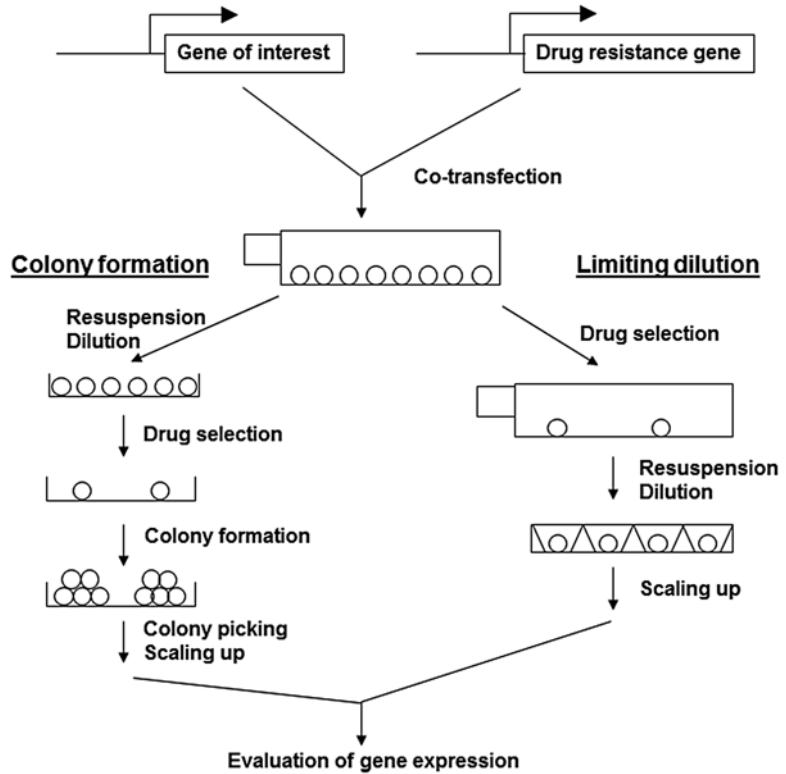
Fig. 1 An overview of two methods for generating monoclonal isolates of transformed lepidopteran insect cells. Cells are co-transfected with IE-1 expression plasmids encoding the gene(s) of interest and a selectable drug resistance marker. After drug selection, clonally derived cell lines can be isolated either by picking and expanding colonies formed on petri dishes or by growing lines from individual drug-resistant cells deposited into the wells of 96-well plates

2   Materials

1. Cell lines: Sf-9 (ATCC: #CRL-1711; *see* **Note 2**) can be obtained from the American Type Culture Collection (Manassas, VA 20108; www.atcc.org) or from Life Technologies (Carlsbad, CA; www.lifetechnologies.com).

2. Cell media: Grace's Insect Medium, Hink's TNM-FH medium, and Pluronic® F-68 are available from Sigma-Aldrich Corporation (St. Louis, MO; www.sigma-aldrich.com; *see* **Note 3**).

3. Plastics: Falcon and Corning flasks, petri dishes, and multiwell plates for cell culture are available from Fisher Scientific (Pittsburgh, PA; www.fishersci.com).

4. Hemacytometer and cell counters: available form Fisher Scientific.

5. Fetal bovine serum is available from Life Technologies (*see* **Note 4**).

6. Conventional cell culture antibiotics: Amphotericin B (Fungizone®) and gentamicin can be obtained from Life Technologies.

7. Antibiotics for selection of transformed cells: G418 (Geneticin®), hygromycin B, Zeocin™, and blasticidin S are available from Life Technologies.

8. Enzymes for constructing expression plasmids: Restriction endonucleases, calf intestinal alkaline phosphatase, DNA polymerase I (Keno fragment), and T4 DNA polymerase can be obtained from New England Biolabs (Ipswich, MA; www.neb.com).

9. Plasmids: The pIEHR series of expression vectors (Fig. 2), pDIE1HR1 (Fig. 1, Chapter 18), pDIE1-(fluorescent protein)-TOPO.4 series of expression vectors (Fig. 1, Chapter 18), and the selection plasmids pIE1Neo and
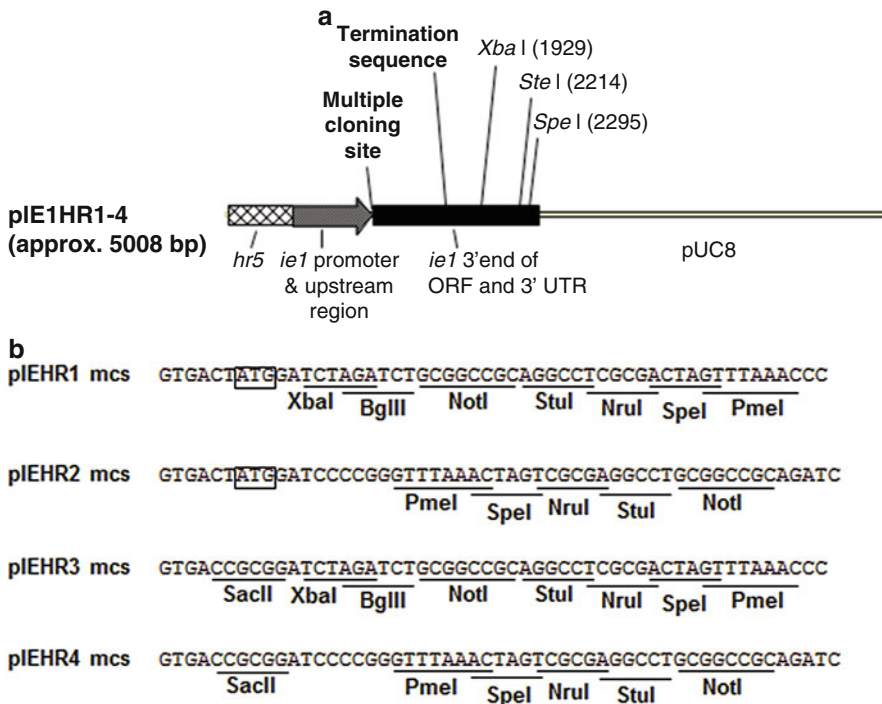

Fig. 2 (**a**) Features of the pIE1HR series of plasmids used for transformation and constitutive gene expression in lepidopteran insect cells. The *hr5* enhancer, *ie-1* promoter and termination sequence, and multiple cloning site are indicated. Restriction sites that are also present in the multiple cloning sites are noted. (**b**) Sequences of the multiple cloning sites for the pIE1HR series (1–4) with restriction sites *underlined*. pIE1HR1 and pIEHR2 utilize the native *ie-1* start codon (*boxed* ATG) for translation initiation. In pIE1HR3 and pIE1HR4, the *ie-1* start codon has been replaced with a *Sac* II site

pIE1Hygro are available from Dr. Donald Jarvis upon request. A series of vectors that use the same *hr5-ie-1* enhancer-promoter arrangement (the pIEx™ series) is available for purchase from EMD Millipore (Billerica, MA; www.emdmillipore.com). In addition, Life Technologies sells expression plasmids (the pIB and pIZ series) that carry drug resistance genes for blasticidin S and Zeocin™.

10. Chemicals: Buffers (HEPES, Tris), NaCl, CaCl$_2$, ethylenediaminetetraacetic acid (EDTA), 100 % ethanol, ethidium bromide, glucose, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), sodium acetate, potassium acetate, potassium hydroxide, formic acid, glacial acetic acid, Tris-equilibrated phenol, and chloroform can be obtained from Fisher Scientific. Molecular biology-grade reagents should be purchased when available.

11. Deionized, distilled, DNAse- and RNAse-free water: If a water purification system and an autoclave are not available, bottles of sterile water suitable for molecular biology research can be purchased from Fisher Scientific.

12. Benchtop pH meter: Several brands are available from Fisher Scientific.

13. Solutions for vector preparation, miniprep and maxiprep DNA isolation and purification, and cell transfection: 1:1 phenol–chloroform, with phenol pre-equilibrated to pH 8.0 with Tris buffer; 3 M sodium acetate, brought to pH 5.2 with glacial acetic acid; Solution I (50 mM glucose, 10 mM EDTA, and 25 mM of a Tris–HCl, pH 8.0, which is prepared from a Tris buffer stock solution brought to pH 8.0 with HCl); Solution II (1.0 % sodium dodecyl sulfate, 0.2 N NaOH); Solution III (3 M potassium acetate, 1.8 M formic acid); TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA); Transfection buffer (140 mM NaCl, 125 mM CaCl$_2$, and 25 mM HEPES, pH 7.1, prepared from a HEPES stock brought to pH 7.1 with potassium hydroxide).

14. Liposome transfection reagents: Cellfectin® II Reagent can be obtained from Life Technologies.

15. Cloning reagents (bacteria): Competent *E. coli* are available from Promega (Madison, WI; www.promega.com).

16. Cloning reagents (bacteria media, etc.): LB broth, 2×YT broth, LB agar, ampicillin, and 100 mm plastic petri dishes for agar plates are available from Fisher Scientific.

17. Additional equipment for vector preparation: Vortex mixers, microcentrifuges, standard laboratory incubators and incubating shakers capable of maintaining 37 °C, and UV-Vis spectrophotometers are available from Fisher Scientific. Floor model centrifuges, ultracentrifuges, rotors, and other centrifuge

accessories are available from Thermo Scientific (Waltham, MA; www.thermoscientific.com) or Beckman Coulter (Brea, CA; www.beckmancoulter.com)

18. Micropipetters can be obtained from Fisher Scientific or Mettler Toledo (Greifensee, Switzerland; us.mt.com). Pasteur pipettes and vacuum grease are available from Sigma-Aldrich Corporation. Glass cloning cylinders can be obtained from Bellco Glass, Inc. (Vineland, NJ; www.bellcoglass.com).

3   Methods

3.1   Insertion of the Gene of Interest into an Expression Plasmid

Prior to cell transformation, the gene of interest must be placed into a plasmid under the control of a promoter that is constitutively active in uninfected cells. We routinely use a set of plasmids, designated "immediate early expression plasmids" in which the gene of interest is inserted between the AcMNPV *ie-1* promoter/upstream region and the *ie-1* transcriptional termination signal. The *ie-1* promoter is linked in *cis* to the AcMNPV *hr5* enhancer (Fig. 2) [10]. This enhancer stimulates *ie-1*-mediated transcription in transfected or transformed insect cells when these cells are infected with a baculovirus or co-transfected with a plasmid encoding the baculovirus IE1 protein [7, 33]. These four immediate early expression plasmids differ in the arrangement and selection of restriction sites available for inserting the gene of interest and also by the presence or absence of the *ie-1* start codon. pIE1HR1 and pIE1HR2 both include the native *ie-1* start codon upstream of the multiple cloning site. These plasmids were originally designed to express genes of interest without their own ATGs, after being inserted in-frame with the upstream, native translational initiation site. Conversely, the *ie-1* ATG was replaced with a *SacII* site in pIE1HR3 and pIE1HR4 site. Hence, these vectors were designed to express genes of interest with their own ATGs, which can be inserted without considering the open reading frame. We have also constructed plasmids capable of expressing two genes at once. These "dual" immediate early expression plasmids consist of a single copy of *hr5* flanked by two copies of the *ie-1* promoter oriented in opposite directions [16, 34, 35] (also *see* Fig. 1 and Chapter 18). This expression plasmid can be used to produce a cell line that expresses two genes of interest for various applications, including simultaneous production of both partners of a heterodimer.

None of the expression plasmids described above have selectable markers. Thus, to isolate transformed insect cells, we routinely co-transfect with a separate selection plasmid that encodes a neomycin-(pIE1Neo) [6] or hygromycin B-(pIE1Hygro) [21] resistance marker. The following is an outline of the steps we use to insert a foreign gene into one of the expression vectors detailed in

Fig. 2, and to produce a preparation of the resulting plasmid that can be used in transfecting insect cells.

 1. Digest one of the pIEHR series of vectors with restriction enzymes that cut at the sites needed to insert the gene.

 2. Dephosphorylate the ends with calf intestinal alkaline phosphatase. If the restriction fragment carrying the gene of interest has blunt termini, then blunt the ends of the vector with the Klenow fragment of *E. coli* DNA polymerase I (for recessed 3′ ends) or T4 DNA polymerase (for the protruding 3′ end produced by cleavage with *Sac* II).

 3. Bring the vector preparation volume to 500 μL with distilled water. Add 500 μL of a 1:1 mixture of phenol (equilibrated to pH 8.0) and chloroform. Vortex briefly and separate the organic and aqueous phases by microcentrifugation ($12,500 \times g$ for 5 min). Transfer the aqueous (top) phase to a fresh tube and add 50 μL 3 M sodium acetate (pH 5.2) and 1 mL 100 % ethanol. Vortex briefly and pellet precipitated DNA by microcentrifugation ($14,000 \times g$ for 15 min).

 4. Prepare the gene of interest as a restriction fragment. Run aliquots of the vector and the restriction fragment carrying the gene of interest on an agarose gel to evaluate the relative quantities of each.

 5. Set up a ligation reaction with the gene of interest present at a 2:1 or greater molar excess over the vector.

 6. Transform competent *E. coli* cells with the ligation products. Spread transformed cells on LB-agar plates containing 50 μg/mL ampicillin and incubate plates overnight at 37 °C to allow for colony growth.

 7. Set up test tubes containing 2.5 mL of 2× YT medium with 50 μg/mL ampicillin. Inoculate each tube with a colony. Shake tubes at 225 rpm 8 h to overnight at 37 °C.

 8. Pipette 1.5 mL of each culture into individual microfuge tubes. Pellet bacteria by brief microcentrifugation ($14,000 \times g$ for 1 min). Pour off supernatant, drain the pellets, and place tubes on ice.

 9. Isolate plasmid DNA from the pellets by the alkaline lysis method [36]. To start, add 100 μL Solution I. Vortex to resuspend the pellets.

10. Add 200 μL Solution II to each tube. Mix by inversion. The contents of the tubes should be viscous.

11. Add 150 μL Solution III and mix by inversion. A white flocculent precipitate should appear.

12. Pellet the precipitate by microcentrifugation ($14,000 \times g$ for 5 min). Transfer the supernatant to a fresh Eppendorf tube.

Extract the plasmid DNA with phenol–chloroform and precipitate with ethanol as described in **step 3**.

13. Resuspend DNA pellets in 50–100 μL deionized, distilled H_2O. Screen the plasmids for the presence and proper orientation of the inserted gene by restriction mapping.

14. Use 0.5 mL of leftover miniprep culture from an appropriate clone to inoculate 200 mL of 2× YT containing 50 mg/mL ampicillin in a 500 mL flask. Incubate with shaking (225 rpm) overnight at 37 °C.

15. Pellet bacteria by centrifugation ($5000 \times g$ for 10 min). Decant supernatant and resuspend pellets in a total of 4 mL Solution I.

16. Add 8 mL Solution II and incubate on ice with gentle shaking for 5 min. Add 6 mL Solution III and shake again on ice for 5 min.

17. Pellet flocculent material by centrifugation ($10{,}000 \times g$ for 15 min).

18. Transfer supernatant to a 50 mL conical tube. Fill tube with 100 % ethanol, mix by inversion, and pellet nucleic acid by centrifugation ($5000 \times g$ for 15 min). Decant supernatant and drain pellet.

19. To form CsCl-ethidium bromide equilibrium density gradients, resuspend the pellet in a volume of TE appropriate for the type of tube to be used for ultracentrifugation. Add 1.01 g CsCl per g of suspended pellet and 100 μL 10 mg/mL ethidium bromide per 5 g of suspended pellet. Vortex to dissolve the CsCl. Transfer suspension to a tube suitable for centrifugation in an ultracentrifuge rotor.

20. Centrifuge the tubes at 20 °C at a relative centrifugal force appropriate to the rotor [37]. For example, if using a Beckman Type 70.1 Ti rotor, the tubes should be centrifuged at $331{,}000 \times g$ for 24 h.

21. After centrifugation, collect the band of closed circular plasmid DNA from the gradients and transfer to a 15 mL conical tube. To remove ethidium bromide from the plasmid DNA, add an equal volume of water-saturated butanol and vortex. Allow the aqueous and butanol phases to separate. Remove the top (butanol) phase and discard. Repeat the extraction until the top phase is clear.

22. Transfer DNA to a 50 mL conical tube, dilute fivefold with deionized, distilled H_2O, and precipitate the DNA with 100 % ethanol. Pellet DNA and resuspend in TE pH 8.0.

23. Quantitate the DNA by measuring absorbance of a dilution in TE at 260 nm and verify its structure by restriction endonuclease mapping.

3.2   Transfection   As outlined in Fig. 1, cells are co-transfected with a mixture of an expression plasmid bearing the gene of interest and another carrying a drug resistance gene. The following protocol describes the steps for co-transfection of Sf-9 cells using the calcium phosphate precipitation method [38] prior to antibiotic selection. An alternative method is transfection with DNA packaged into liposomes formed by a commercial cationic lipid reagent (such as Cellfectin® II from Life Technologies), which should be done following the vendor's recommendations. Both methods result in an efficiency of transfection sufficient for producing stably transformed cells, but it has been reported that liposome-mediated transfection results in a lower number of copies of the expression vector integrated into the cells' chromosomes [39].

1. Seed each of two 25 cm² T-flasks with 2×10^6 Sf-9 cells in a final volume of 5 mL of antibiotic-free medium. One of the flasks will be transfected in the presence and the other in the absence of the selectable marker to verify the efficacy of the selection process. The cells must be healthy (97–98 % viability) and grown in the absence of antibiotics.

2. Allow cells to attach for 1 h.

3. Prepare the plasmid DNAs for transfection as follows:

   (a) Place 1 μg of the selectable marker and 2–20 μg of the immediate early expression plasmid(s) encoding the protein(s) of interest into a single microfuge tube (*see* **Note 5**). Place the same amount of the immediate early expression plasmid(s) encoding the protein(s) of interest, with no selectable marker, into a second microfuge tube.

   (b) Ethanol-precipitate to sterilize the DNAs and prevent bacterial contamination of the transfections. Use 70 % ethanol to surface-sterilize the microfuge tubes, as well.

   (c) Aseptically transfer 0.75 mL of transfection buffer to the tubes containing the DNA pellets and gently resuspend the DNAs in this buffer.

4. Remove the TNM-FH medium from both cell cultures. Rinse twice with 5 mL of complete Grace's medium (Grace's medium supplemented with 10 % fetal bovine serum, 1.25 μg/mL amphotericin B, and 25 μg/mL gentamycin). Remove the last rinse from the flasks completely and add 0.75 mL of complete Grace's medium to each flask.

5. Add the DNA mixtures, dissolved in transfection buffer (*see* **Note 6**), to the appropriate flasks. Manually rock the flasks from side to side briefly and gently to mix the transfection buffer and Grace's medium.

6. Incubate the flasks at 28 °C for 2 h. The transfection mixture should take on a milky white appearance. If this does not happen, then check the pH of the transfection buffer.

7. Drain the transfection mixtures from the cells. Rinse twice with complete TNM-FH medium (TNM-FH supplemented with 10 % fetal bovine serum and antibiotics).

8. Feed the cells with 5 mL complete TNM-FH and incubate overnight at 28 °C.

3.3 Selection and Isolation of Transformed Clones by the Colony Formation Method

This procedure takes advantage of the propensity of insect cells to form discrete colonies when cultured at extremely low densities. Selection and amplification of transformed cell clones requires long-term culture (approximately 1 month). It is therefore critical to be extremely meticulous to avoid microbial contamination.

1. Dislodge cells from the surface of the flasks obtained in Subheading 3.2, **step 8**. We routinely use the medium in the flask and a Pasteur pipette to squirt the cells off the plastic surface ("fire-hose" method). An alternative method for dislodging cells is given in Chapter 9.

2. Dilute each culture to a total volume of 30 mL with complete TNM-FH.

3. Set up 60-mm petri dishes with 3 mL complete TNM-FH. Plate diluted cells using the split ratios given below, depending upon the amount of expression plasmid used for transfection (*see* **Note 7**):

   (a) For 2 μg of DNA, seed at 1:60 and 1:75

   (b) For 5–10 μg of DNA, seed at 1:45 and 1:60

   (c) For 20 μg of DNA, seed at 1:30 and 1:45

4. Seal the dishes in a humidified plastic baggie to minimize evaporation of the medium. Incubate the dishes overnight at 28 °C.

5. Replace the medium with complete TNM-FH containing the selection antibiotic (4 mL per 60-mm dish; *see* **Note 8**).

6. Incubate the dishes at 28 °C for 1 week. After 3–4 days, all the control cells transfected with the expression plasmid alone should be dead (*see* **Note 9**). If the control cells are still alive after 1 week of antibiotic treatment, then the selection procedure has failed.

7. Remove the old medium, wash gently with complete TNM-FH, and add fresh complete TNM-FH plus the selection antibiotic. Incubate for another week at 28 °C.

8. Remove the old medium, wash cells gently with complete TNM-FH, and add fresh complete TNM-FH without any antibiotic. Incubate dishes at 28 °C until large colonies of densely packed cells form. The colonies should be at least 2 mm in diameter. Smaller, relatively less dense colonies are unlikely to survive the cloning procedure (*see* **Note 10**). Larger colonies are likely to be lost by detaching from the surface of the plate.

9. Drain one 60-mm dish to pick the colonies. Do not drain more than one dish at a time, as the cells will dry out in the additional dishes and die before you can pick the colonies.

10. Using forceps, dip a cloning cylinder in alcohol and flame-sterilize it. Dip one end of the sterilized cylinder into a dollop of vacuum grease on a petri dish. Place the cylinder, greased-end down, onto the drained dish around a well-isolated colony. The vacuum grease must form a seal between the cylinder and the dish.

11. Add 100 µL of complete TNM-FH to the interior of the cloning cylinder and use a micropipettor to gently disperse the cells in the colony. Transfer the dispersed cells to a single well in a 96-well plate.

12. Monitor the cells daily. The cells may grow to 80–90 % confluency in 3–7 days. Approximately half the colonies will fail to grow. Amplify each surviving colony in stepwise fashion by transferring the cells first to a 24-well plate, then to a 6-well plate, and then to a 25-cm² flask. Allow the cells to grow close to confluency at each step.

13. When the 25-cm² flask culture is nearly confluent, split it 1:3. Use two of the resulting 25-cm² flasks, which we define as passage 1, to prepare P1 freezer stocks (*see* **Note 11**). Use the remaining P1 culture for screening and further amplification.

3.4 Selection and Isolation of Transformed Clones by the Limiting Dilution Method

This procedure is a more reliable way to obtain verified, single cell clones than the colony-formation method. However, it is unclear whether all established insect cell lines are amenable to single cell cloning by the limiting dilution method.

1. Drain media from the flasks from **step 8** of the co-transfection procedure (Subheading 3.2) and add 5 mL complete TNM-FH plus selection antibiotic.

2. Incubate flasks 1 week at 28 °C. All the cells in the control flasks should be dead. Many cells in the flasks co-transfected with the expression plasmid and the resistance marker will also die, but there should be a significant number of surviving cells.

3. Use the fire-hose method to detach cells from the flasks and perform a cell count.

4. Dilute the cells to a concentration of about 5 cells/mL with Sf-9 cell-conditioned medium (medium from Sf-9 exponential growth phase culture; *see* Chapter 1).

5. Dispense 200 µL of the diluted cells into multiple wells of a 96-well plate. Check with a phase-contrast microscope to determine if most wells contain a single cell. If this is not the case, then adjust your dilution as necessary and try again. Once you find the appropriate dilution, dispense enough cells to fill

an entire 96-well plate because about half of the single cell clones will probably fail to grow.

6. Monitor the wells daily for cell growth. As the clones grow, amplify each one stepwise as described in **step 12** under Subheading 3.3.

7. Process the 25-cm² flask, P1 cultures as described in **step 13** of the colony formation method (Subheading 3.3).

3.5 Screening for the Presence and Expression of the Gene of Interest

Once multiple antibiotic-resistant insect cell clones have been isolated, they must be screened to determine if they contain and express the gene of interest. Southern blotting of restriction enzyme-digested genomic DNA from the transformed cells will reveal the presence of the gene of interest integrated into cellular DNA [5]. A simple dot-blot hybridization of cytoplasmic RNA from the transformed cells [40] will reveal whether or not the gene is transcribed, while Northern blotting [41], S1 nuclease [42], primer extension [43], RNAse protection assays [44], and/or rapid amplification of cDNA ends (RACE) [45] can be used to examine transcription of the gene of interest in more detail. If an antibody is available against the protein of interest or an epitope tag fused to the protein of interest [46], then many standard techniques, such as indirect immunofluorescence [47], immunoprecipitation [5], and Western blotting [48], can be used to detect the gene product in transformed cells. These techniques also can provide some information about the localization of the protein in transformed cells. Obviously, none of these detection methods guarantee that the transformed cells are producing an active, biologically functional protein of interest. Hence, depending on the application, it can be critically important to perform a functional assay of transformed insect cell clones containing and expressing the gene of interest. Finally, as mentioned above, it should be recognized that transformed insect cell clones are not guaranteed to be genetically stable, so it is important to monitor chosen clones for retention of the gene of interest as those cells are maintained through increasingly higher passages in the lab.

4   Notes

1. A number of studies indicate that stably transformed insect cells can produce higher quality recombinant proteins and circumvent some difficulties associated with the conventional baculovirus-infected cell approach, particularly for functional analyses of secretory pathway proteins. Baculoviruses produce a cysteine protease during infection that can degrade foreign proteins and further reduce the yield of secreted protein [49, 50]. A comparative study of β1,3-N-acetylglucosaminyltransferase

2 (β3GnT2) expression by Tn-5 cells infected with a baculovirus vector or stably transformed with the β3GnT2 gene [51] revealed that the infected Tn-5 cells produced extensively degraded β3GnT2, whereas the stably transformed Tn-5 cells produced intact β3GnT2. (Note that Tn-5 is the *Trichoplusia ni* BTI Tn-5B1-4 cell line [52] commercially available from Life Technologies as High Five™ cells). Furthermore, the specific activity of β3GnT2 purified from the stably transformed Tn-5 cells was 1.6- to 2.9-fold higher than that purified from the baculovirus vector-infected cells. Another study showed that patch clamp analysis of Sf-9 cells expressing the bovine GABA$_A$ receptor was significantly easier to perform on cells stably transformed with the GABA$_A$ receptor gene than on cells infected with a baculovirus vector encoding the GABA$_A$ receptor [53]. Finally, the human β2-$_a$drenergic receptor expressed in stably transformed Sf-9 cells was able to function in concert with the endogenous insect cell adenylyl cyclase system, which was not observed when the β2-adrenergic receptor was expressed using a standard baculovirus vector [54].

While the above studies demonstrate that the stably transformed cell approach often presents advantages over the infected-cell approach, we also should note that this is not always the case. In a study comparing expression of human estrogen receptor beta (ERβ) in stably transformed IPLB-Sf21-AE (Sf-21) [55] cells versus baculovirus-infected Sf-21 cells, the yield of active ERβ from the infected cells was tenfold higher than that obtained with stably transformed cells expressing ERβ under *ie-2* control [56]. The stably transformed ERβ-expressing Sf-21 cell line exhibited reduced cell growth and viability relative to untransformed Sf-21 cells, suggesting a possible explanation for the superior expression of active ERβ with a baculovirus vector in this instance.

2. For transformations, we usually start with the Sf-9 subclone of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE [55]. We maintain Sf-9 cells in suspension culture in TNM-FH medium supplemented with 10 % (v/v) fetal bovine serum and 0.1 % (w/v) Pluronic® F-68 [57]. For cell transformation, we only use Sf-9 cells that have never been exposed to antibiotics. This practice follows from the observation that Sf-9 cells maintained in 1.25 μg/mL amphotericin B and 25 μg/mL gentamycin were highly resistant to neomycin selection [28]. We also have successfully transformed Tn-5 cells [52] using the procedures described herein, though we were unable to isolate single cell clones by limiting dilution in that particular study [34].

3. Grace's and TNM-FH media also can be prepared according to published recipes [38]. All media and media components should be stored in the dark at 4 °C.

4. Although heat inactivation of serum to be added to insect cell medium has been a standard practice, we no longer heat-inactivate the serum used in our growth media because studies performed at Thermo Scientific HyClone have shown that heat inactivation has no positive influence on insect cell culture (*see* "Heat Inactivation—Are You Wasting Your Time?" in *Art to Science in Tissue Culture*, Vol. 15, No. 1). Different batches of serum differ in their capacity to support insect cell growth so it is important to test new serum lots before making a large-scale purchase.

5. Within the range of 2–20 μg of expression plasmid, there does not appear to be any significant difference in the transformation efficiencies or expression levels obtained with transformed clones produced by G418 selection. In fact, there are typically fewer total G418-resistant colonies obtained with larger amounts of expression plasmid. This is why lower split ratios are used to isolate clones in **step 3** under Subheading 3.3 after transfection with higher quantities of expression plasmid.

6. The pH of the transfection buffer can drastically affect the efficiency of transfection and must be adjusted precisely. After preparation, the transfection buffer must be filter-sterilized and stored at 4 °C.

7. Because the cells in the flask are suspended in a total of 30 mL medium, seeding 1 mL of this suspension into a dish yields a 1:30 split. Higher split ratios are achieved by seeding less than 1 mL or performing further dilutions with complete TNM-FH. To ensure that well-isolated colonies are obtained, a range of split ratios should be used (1:20–1:100).

8. We have routinely used G418 and hygromycin B at a concentration of 1.0 mg/mL and also have used Zeocin™ at sequentially increasing concentrations from 0.3 to 1.0 mg/mL. After addition of the antibiotic, the medium is filter-sterilized and supplemented with 10 % fetal bovine serum. Conventional cell culture antibiotics (1.25 μg/mL amphotericin B and 25 μg/mL gentamycin) also can be added at this time to prevent contamination.

9. If the control cells survive antibiotic treatment, then (a) determine if the cells were previously exposed to antibiotics and (b) check the expiration date of the antibiotic stock used for selection.

10. Hints for successfully picking and amplifying transformed colonies:

    (a) Small, less dense colonies will not grow. However, colonies that are too large will detach from the surface of the dish and be lost when the growth medium is removed.

(b) Vacuum grease does not seem to create a significant contamination problem. However, one can surface-sterilize the grease by placing a dollop on a petri dish under UV light in a laminar flow hood.

(c) To avoid having the colonies on a plate dry out after removal of growth medium, place cloning cylinders over no more than four colonies before adding medium to the interior of each cylinder.

(d) To obtain single cell suspensions, triturate individual colonies with a micropipettor. If the cells are still clumpy after being seeded into a 96-well plate, then they can be dispersed by additional trituration. Examine the plates with a phase-contrast microscope after picking colonies to make sure that most of the cells from each colony have been collected. If not, then add more medium and use a micropipettor to recover the additional cells.

(e) We typically pick 12 antibiotic-resistant colonies of cells transformed with a single gene of interest. Some colonies will stop growing, grow slowly, or become contaminated during the amplification process. However, most of the survivors typically contain and express the unselected marker. It is typically necessary to start with larger numbers of antibiotic-resistant colonies (or single cell clones) to isolate transformed clones containing and expressing multiple unselected markers.

11. Freezer stocks are prepared by gently removing cells from the 25-cm² flask surface, pelleting the cells by low-speed centrifugation (approximately $120 \times g$) for 1 min, and resuspending in complete TNM-FH plus 10 % DMSO. Optimal cell cryopreservation and recovery is achieved with a cooling rate of –1 °C/min. The Cryo 1 °C Freezing Container ("Mr. Frosty") from Nalgene (Rochester, NY; catalog #5100-0001) provides this cooling rate. Subsequently, the cells should be placed in liquid nitrogen for long-term storage.

Acknowledgments

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