UNIT 16.9

Preparation of Insect Cell Cultures and Baculoviral Stocks

NOTE: Use sterile technique for all procedures in this unit.

BASIC PROTOCOL

MAINTENANCE AND CULTURE OF INSECT CELLS

Materials (see APPENDIX 1 *for items with ✓)*

✓ Complete baculoviral medium, room temperature
Spodoptera frugiperda (*Sf*9) cells derived from fall armyworm ovaries (frozen ampule; ATCC #CRL 1711)
70% ethanol
0.4% trypan blue in PBS, filter-sterilized (optional)
Complete medium containing 10% DMSO, ice-cold

25-cm^2 flasks
37°C water bath and 27°C incubator
Hemacytometer for tissue culture cells
Spinner culture flasks (for suspension cultures; Bellco)
Screw-top cryostat freezing vials
Liquid nitrogen freezer

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Begin culture of Sf9 cells

1. Place 4 ml complete medium in a 25-cm^2 flask.
2. Thaw frozen ampule of *Sf*9 cells rapidly in 37°C water bath. When contents of ampule are almost completely thawed, immerse ampule in 70% ethanol to sterilize the outside. Break neck of ampule and transfer its contents to 25-cm^2 flask with 4 ml complete medium. Rock flask gently by hand to distribute cells evenly and incubate at 27°C.
3. Allow cells to attach 2 to 3 hr, remove old medium, and replace with 5 ml fresh complete medium. Feed every 3 days until cells reach confluency.

Maintain and subculture monolayer cultures

4. Prepare new flask by placing 4 ml complete medium in 25-cm^2 flask (or use larger flask with more medium if preparing larger culture of cells). Remove medium from confluent flask of *Sf*9 cells (step 3), and resuspend cells in fresh complete medium.
5. Count cells using hemacytometer designed for tissue culture cells. Each cell in a small square is equivalent to 10^4 cells/ml.
6. Seed 1-2 × 10^6 cells into new 25-cm^2 flask. Incubate at 27°C. Feed culture every 3 days with complete medium until cells reach confluency.

Maintain and subculture suspension cultures

7. Remove medium and resuspend cells from confluent monolayer culture as in step 4. Count cells using hemacytometer as in step 5.
8. Seed cells into spinner culture flask at ~5 × 10^5 cells/ml. Incubate at 27°C with constant stirring at ~50 rpm.
9. Count cells every 3 days. Subculture when cells reach a concentration of 2-2.5 × 10^6 cells/ml to a new flask containing fresh medium for a

final density of 5×10^5 cells/ml. (Alternatively, pour out appropriate volume of cell suspension and replace with fresh complete medium.) Concentrate cells by centrifugation, resuspended in fresh medium, and transfer to new spinner flask at least once per week to keep cells healthy.

To determine cell viability, add 0.1 ml of 0.4% trypan blue in PBS, filter sterilized, to 1 ml log-phase cells. Examine cells under microscope at low power. Count number of cells that take up trypan blue (dead cells) and count total number of cells. A healthy culture of cells should be ≥97% viable.

Freeze cells

9. Count cells to be frozen from an exponentially growing culture using hemacytometer (step 4).
10. Centrifuge 2×10^7 cells 10 min at $1000 \times g$, room temperature. Rinse cells in fresh complete medium and centrifuge again.
11. Resuspend cell pellet (on ice) to $2\text{-}4 \times 10^6$ cells/ml in ice-cold complete medium containing 10% DMSO. Dispense cells into screw-top cryostat freezing vials (~5 to 10 vials) and place cells 2 hr at −20°C, then overnight at −80°C. Transfer frozen cells to liquid nitrogen freezer for long-term storage the following day.

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PREPARATION OF BACULOVIRAL STOCKS

BASIC PROTOCOL

Materials (see APPENDIX 1 *for items with ✓)*

*Sf*9 cells (first basic protocol)
✓ Complete baculovirus medium *without* FBS
Autographa californica multiply-enveloped nuclear polyhedrosis virus, wild-type [AcMNPV titered as pfu/ml; available from M.D. Summers, *UNIT 16.8*, or Invitrogen (MaxBac)]
150-cm² flasks
100- or 200-ml spinner culture flasks (Bellco)

From monolayer cultures:

1a. Seed two 150-cm² flasks with 1.8×10^7 *Sf*9 cells/flask. Allow cells to attach for 3 hr, then remove complete medium. Add 5 ml serum-free complete medium supplemented with wild-type virus at a multiplicity of infection (MOI) of 0.1. Incubate 1 hr at 27°C.

2a. Remove viral inoculum and replace it with 20 ml complete medium. Incubate cells at 27°C. Examine cells daily under light microscope for signs of occlusion bodies, which usually appear after 2 to 4 days (occlusion bodies are highly refractile; see *UNIT 16.10*).

3a. Harvest virus when majority of cells contain occlusion bodies by removing infected culture medium to sterile tubes. Centrifuge 10 min at $1000 \times g$, 4°C, and transfer supernatant to new, sterile tubes. Dispense 1-ml aliquots (~40 ml at $10^8\text{-}10^9$ pfu/ml) into several screw-top cryostat freezing vials and place in liquid nitrogen freezer for long-term storage. Store remaining stock at 4°C.

From suspension cultures:

1b. Grow *Sf*9 cells in 100-ml spinner culture flask with 50 ml complete medium to $\sim 2 \times 10^6$ cells/ml with constant stirring. Centrifuge 10 min

at 1000 × g room temperature. Resuspend cell pellet in 10 to 20 ml serum-free complete medium supplemented with wild-type virus at an MOI of 0.1, and incubate 1 hr at room temperature.

2b. Bring volume to 100 ml with complete medium and place cells in 200-ml spinner culture flask or in two 100-ml spinner flasks. Incubate 2 to 4 days at 27°C with constant stirring.

3b. Harvest infected cells and store as described in step 3a.

SUPPORT PROTOCOL

TITERING BACULOVIRAL STOCKS USING PLAQUE ASSAYS

Additional Materials (see APPENDIX 1 for items with ✓)

✓ Agarose overlay (prepare 30 min before use; step 4)
60-mm Lux tissue culture plates (Nunc Intermed #5220)

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1. Seed cells into 60-mm Lux tissue culture plates at 2×10^6 and 1.5×10^6 cells/plate several hours before plaquing. Set up duplicate plates for each dilution of viral stock. Place at 27°C.

   The density of cell monolayer is critical when plaquing virus; if cells are too dense, plaques will not break through the monolayer to form a visible hole, and if they are too sparse, it will be difficult to distinguish plaques from areas where the cells have not filled in.

2. When ready to plaque, make 5-ml serial dilutions of viral stocks in serum-free complete medium as follows: pure viral stock—10^{-7}, 10^{-8}, and 10^{-9} dilutions; transfection supernatant—10^{-4}, 10^{-5}, and 10^{-6} dilutions (*UNIT 16.10*); and single plaque—10^{-1}, 10^{-2}, and 10^{-3} dilutions (*UNIT 16.10*).

3. Remove medium from cells (step 1) with a sterile Pasteur pipet. Add 1 ml of each viral dilution to duplicate plates and incubate 1 hr at room temperature or 27°C.

4. After 30 min prepare agarose overlay.

5. After 1 hr, remove viral supernatant from cells with a sterile Pasteur pipet and add 4 ml agarose overlay. Allow agarose to harden on plates 10 to 20 min at room temperature. Wrap plates individually with Parafilm (to avoid desiccation) and incubate 4 to 8 days at 27°C.

   If plaques are not clearly visible by 1 week, the plates can be incubated longer, since plaques continue to form for up to 2 weeks. If no plaques are visible by 1½ to 2 weeks, the cells were probably plated at too high a density and it is necessary to replaque.

6. On plates containing plaques that are well formed and easily visualized, count number of plaques at each dilution. Calculate titer (pfu/ml).

   If difficulties are encountered visualizing plaques, stain them with trypan blue.

Reference: Summers and Smith, 1987.

Contributor: Helen Piwnica-Worms

SECTION II

EXPRESSION OF PROTEINS IN INSECT CELLS USING BACULOVIRAL VECTORS

UNIT 16.8

16.8

Overview of the Baculoviral Expression System

Baculoviruses have emerged as a popular system for overproducing recombinant proteins in eukaryotic cells for several reasons. First, unlike bacterial expression systems, the baculovirus-based system is eukaryotic and thus uses many of the protein modification, processing, and transport systems present in higher eukaryotic cells. In addition, the baculoviral expression system uses a helper-independent virus that can be propagated to high titers in insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant protein with relative ease. The majority of this overproduced protein remains soluble in insect cells by contrast with the insoluble proteins often obtained from bacteria. Furthermore, the viral genome is large (130 kbp) and thus can accommodate large segments of foreign DNA. Finally, baculoviruses are noninfectious to vertebrates, and the polyhedrin promoter has been shown to be inactive in mammalian cells—an advantage over other systems when expressing oncogenes or potentially toxic proteins.

BACULOVIRUS LIFE CYCLE

Currently, the most widely used baculovirus expression system utilizes a lytic virus known as *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV; hereafter called baculovirus). This virus is the prototype of the family *Baculoviridae*. It is a large, enveloped, double-stranded DNA virus that infects arthropods. The baculoviral expression system takes advantage of some unique features of the viral life cycle (Fig. 16.8.1). See Doerfler and Bohm (1986) for a comprehensive review.

Two types of viral progeny are produced during the life cycle of the virus: extracellular virus particles (nonoccluded viruses) and polyhedra-derived virus particles (occluded viruses). Occluded viral particles are embedded in proteinaceous viral occlusions called polyhedra within the nucleus of infected cells. The polyhedrin protein (29 kDa) is the major protein component of the occlusion bodies. Although the polyhedrin protein is essential for survival of the virus in nature, it is dispensable for virus survival and propagation in tissue culture cells.

BACULOVIRAL EXPRESSION SYSTEM

To produce a recombinant virus that expresses the gene of interest, the gene is first cloned into a special vector (see below). The gene, once cloned into the expression vector, is flanked both 5′ and 3′ by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter. Next, the recombinant vector is transfected along with purified wild-type viral

DNA into insect cells. In a homologous recombination event, the foreign gene is inserted into the viral genome and the polyhedrin gene is excised. Recombinant viruses lack the polyhedrin gene and in its place contain the inserted gene, whose expression is controlled by the polyhedrin promoter.

Homologous recombination occurs at low frequency (1-5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. One of the beauties of this expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein is produced at very high levels in the nuclei of infected cells at late times after viral infection and accumulates to form occlusion bodies that also contain embedded virus particles. These occlusion bodies, ≤15 µm in size, are highly refractile, giving them a bright shiny appearance under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells (*UNIT 16.10*). The plaques are then screened under the light microscope for the presence (wild-type virus) or absence (recombinant virus) of occlusion bodies.

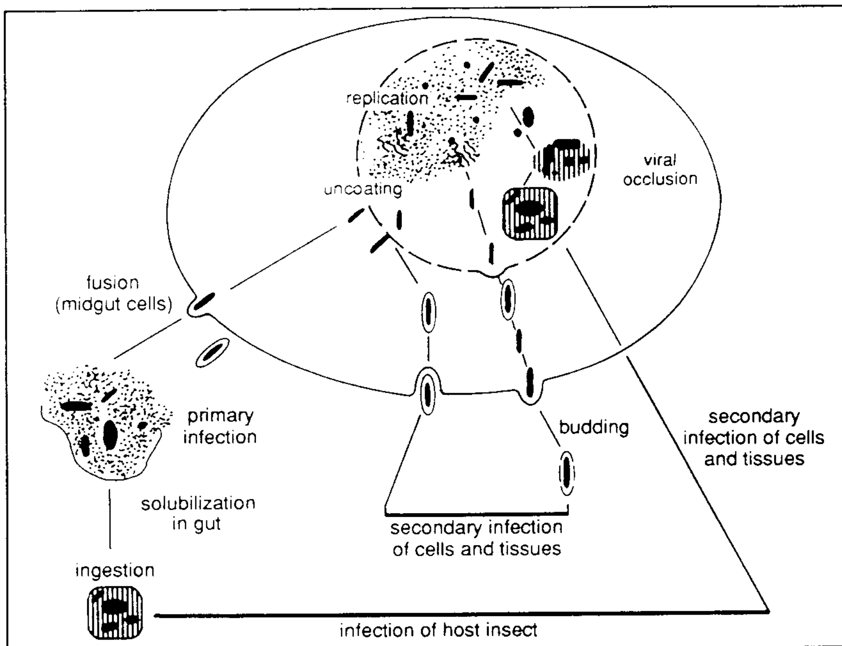

Figure 16.8.1 Baculovirus life cycle. Reproduced from Summers and Smith (1987) with permission from the Texas Agricultural Experiment Station. Viruses enter cells by adsorptive endocytosis and move to the nucleus where their DNA is released. Both DNA replication and and viral assembly take place in the nuclei of infected cells to generate two types of viral progeny. These include extracellular (nonoccluded) virus particles and polyhedra-derived (occluded) virus particles. Extracellular virus is released from the cell by budding, starting at ~12 hr postinfection or until cellular lysis. Polyhedra-derived virus is embedded in proteinaceous viral occlusions, the major protein component of which is the viral polyhedrin protein. Secondary infection of cells and tissues occurs by two pathways. In the first, the extracellular virus, once budded from the site of primary infection, is free to infect neighboring cells by the pathway just described. Alternatively, polyhedra-derived virus is released from occlusion bodies after an infected food source is ingested by a new host.

PROTEIN PROCESSING IN INSECT CELLS

Because baculoviruses infect invertebrate cells, it is possible that processing of proteins produced by them are different from proteins produced by vertebrate cells. While this seems to be the case for some posttranslational modifications, it is not the case for others. For further information on protein processing in insect cells, see Jarvis and Summers (1990) and Luckow and Summers (1988).

STEPS FOR OVERPRODUCING PROTEINS USING THE BACULOVIRAL EXPRESSION SYSTEM

Overproduction of recombinant proteins using the baculoviral system is presented in *UNITS 16.9-16.11*. A brief overview is presented in Fig. 16.8.2.

REAGENTS AND SOLUTIONS FOR THE BACULOVIRAL EXPRESSION SYSTEM

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Commonly used reagents and solutions are summarized below.

1. *Spodoptera frugiperda* (*Sf*9) cells (derived from fall armyworm ovaries) from American Type Culture Collection, accession #CRL 1711.

2. Insect cell culture medium (Grace's Antheraea medium), both 1× and 2× strength in powdered or liquid form, from GIBCO/BRL or JR Scientific. For instructions on media preparation from individual components, see Summers and Smith (1987).

3. Difco lactalbumen hydrolysate and yeastolate from VWR Scientific.

4. Incubator set to 27±1°C; CO_2 is not required.

5. Magnetic spinner flasks from Bellco.

6. Stir plate for multiple spinners (Bellco #7760-06005).

7. Fetal bovine serum (FBS). Obtain and test different lots from a number of suppliers. The lot that promotes the best growth rate and cell viability should be purchased in bulk.

8. SeaPlaque agarose from FMC Bioproducts (#50102).

9. Lux 60-mm tissue culture plates from Nunc Intermed (#5220).

10. Antibiotics (optional)—gentamicin and amphotericin B (Fungizone from Flow Laboratories).

11. Microscope—either an inverted light microscope or a dissecting scope.

12. A kit containing appropriate cloning vectors, manual of methods, and wild-type baculovirus is available from Dr. Max D. Summers, Department of Entomology, Texas Agricultural Experiment Station, Texas A & M University, College Station, TX 77843 (409-845-9730). It is necessary to sign a licensing agreement before kit will be sent. A commercial kit, MaxBac, is also available from Invitrogen (*APPENDIX 4*).

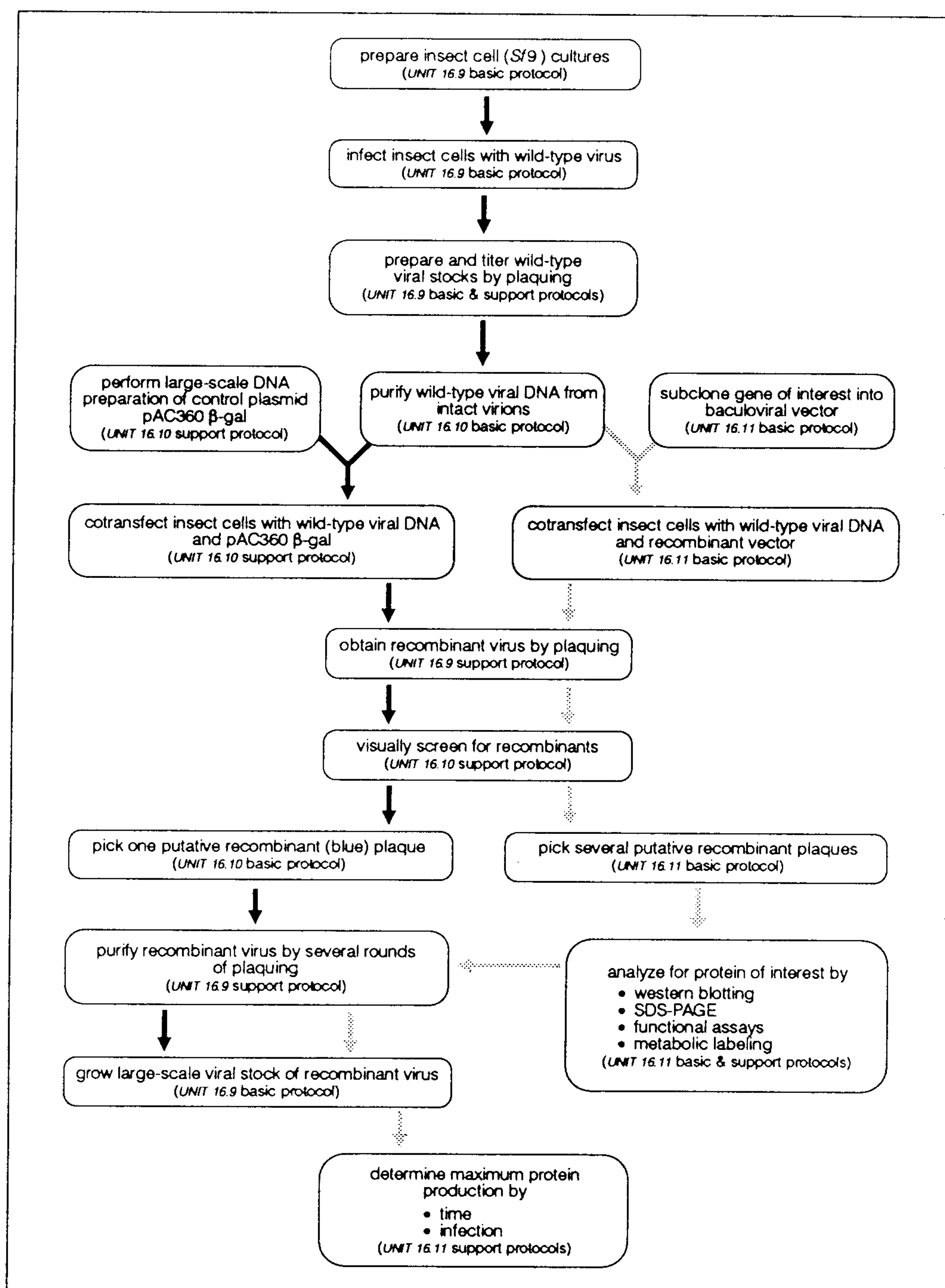

Figure 16.8.2 Flow chart for expression of proteins in insect cells using baculoviral vectors. The black arrows indicate the protocols used to optimize conditions and the light arrows indicate the protocols used to generate recombinant baculoviruses expressing the protein of interest..

References: Doerfler and Bohm, 1986; Jarvis and Summers, 1990; Luckow and Summers, 1988; Summers and Smith, 1987.

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