Generation of Recombinant Baculoviruses and Analysis of the Expressed Proteins

CHOICE OF BACULOVIRAL VECTOR

Available baculoviral vectors are pUC-based and confer ampicillin resistance. Each contains the polyhedrin gene promoter, variable lengths of polyhedrin coding sequence, and insertion site(s) for cloning the foreign gene of interest flanked by viral sequences that lie 5′ to the promoter and 3′ to the foreign gene insert (Table 16.11.1). These flanking sequences facilitate homologous recombination between the vector and wild-type baculoviral DNA. All vectors described below are available from M.D. Summers (*UNIT 16.8*) or Invitrogen (MaxBac). The major consideration when choosing a baculoviral expression vector is whether to express the recombinant protein as a fusion or nonfusion protein in insect cells. The track record for expressing recombinant proteins is good; thus, if the gene contains its own initiation codon, it is preferable to express it as a nonfusion protein.

An improved expression vector that facilitates screening of recombinant baculoviruses has been developed: pBlueBac (Invitrogen), derived from pJV*Nhe*I (Vialard et al., 1990). Because pBlueBac encodes β-galactosidase, the screening of recombinant baculoviruses is simplified (i.e., cotransfection is unnecessary; first basic protocol). pBlueBac has an unique *Nhe*I cloning site and the 5′ polyhedrin mRNA leader sequence of pVL941 is utilized. The vector contains two promoters—the polyhedrin promoter and the p_{10} promoter—both activated very late in viral infection. As with the other baculoviral vectors, the gene of interest is cloned downstream of the polyhedrin promoter, which then controls synthesis of the recombinant protein. The vector also contains the *lacZ* gene positioned downstream of the p_{10} promoter, which directs synthesis of β-galactosidase. The two genes (foreign gene and *lacZ*) and their promoters (polyhedrin and p_{10}) recombine with wild-type virus to yield recombinant virus.

Table 16.11.1 Baculoviral Expression Vectors

Nonfusion expression vector:	Cloning sites:								
pVL941	*Bam*HI								
pVL1393	5′ *Bam*H1 *Sma*I *Xba*I *Eco*RI *Not*I *Xma*III *Bgl*II *Pst*I 3′ (polylinker)								
pVL1392	5′ *Bgl*II *Pst*I *Not*I *Xma*III *Eco*RI *Xba*I *Sma*I *Bam*HI 3′ (polylinker)								
Fusion expression vector:	Cloning sites and sequence:								
pAc700	ATG	GAT	CCG	GTA	CC				
	+1	*Bam*HI		*Kpn*I					
pAc701	ATG	GGA	TCC	GGT	ACC	GAG	CTC		
	+1	*Bam*HI		*Kpn*I					
pAc702	ATG	CGG	ATC	CGG	TAC	C			
	+1	*Bam*HI		*Kpn*I					

CLONING AND SCREENING OF RECOMBINANT BACULOVIRUSES

BASIC PROTOCOL

This protocol provides a summary of all procedures needed to generate a recombinant baculovirus. The appropriate vector should be selected from Table 16.11.1.

1. Subclone gene of interest into appropriate baculoviral vector (*UNIT 3.16*). If pAc700, pAc701, or pAc702 are used, be sure to subclone in the correct reading frame. Select transformants on LB/ampicillin plates (*UNIT 1.1*). Prepare DNA by miniprep procedures and determine which colony is correct clone (*UNIT 1.6*).
2. Pick one colony for large-scale plasmid preparation using alkaline lysis method, including a CsCl/ethidium bromide equilibrium centrifugation (*UNIT 1.7*). Determine vector DNA concentration (*APPENDIX 3*).
3. Prepare for cotransfection by first seeding 2.5×10^6 cells into each of two 25-cm^2 flasks. Allow cells to attach ≥3 hr while warming transfection buffer and $CaCl_2$ solution to room temperature (first support protocol, *UNIT 16.10*).
4. Remove culture medium from flasks and replace with 2 ml Grace's Antheraea medium containing 10% FBS and 50 μg/ml gentamicin. Leave flasks at room temperature.
5. Set up two sterile tubes containing wild-type DNA at optimum concentration as determined in the first support protocol in *UNIT 16.10*. To one tube add 2 μg of pAC360 β-gal (first support protocol, *UNIT 16.10*) and to second tube add 2 μg recombinant baculoviral plasmid (step 2 above). Add 950 μl transfection buffer to each tube and mix. Follow steps 4 to 8 of first support protocol in *UNIT 16.10*.
6. Wait until plaques are well formed (4 to 6 days), then count number of blue plaques (produced by recombinant virus) at each dilution on β-gal-recombinant plates. Recombinant viruses generally comprise 0.1% to 5% of total virus produced. Thus, if there are no blue plaques at the 10^{-4} or 10^{-5} dilutions, retransfect cells (step 3 above).
7. Screen for recombinant viruses as described in second support protocol of *UNIT 16.10*. Start with the 10^{-4} dilution (plate should be covered with ≥1000 plaques). Recombinants are usually found on the 10^{-4} or 10^{-5} dilution plates.
8. Identify and pick several putative recombinant viral plaques. Pick one wild-type viral plaque to be used as a control. Place agarose plugs in 1 ml serum-free medium. Vortex and store at 4°C.

ANALYSIS OF PROTEIN FROM PUTATIVE RECOMBINANT VIRUSES

BASIC PROTOCOL

Before proceeding with further plaque purification, it is recommended that the putative recombinants obtained in the basic protocol above be screened for their ability to produce the protein of interest. The assays employed depend on the nature of the protein being overproduced. This protocol gives some suggested approaches but is certainly not comprehensive. The screen-

ing should be individually tailored to the properties of the protein being overproduced and the availability of detection reagents.

Materials (see APPENDIX 1 for items with ✓)

Spodoptera frugiperda (*Sf*9) cells (ATCC #CRL 1711)
✓ Complete baculoviral medium
✓ Phosphate-buffered saline (PBS)
✓ 1× and 2× SDS/sample buffer
25-cm^2 flasks

1. Seed 2.5 × 10^6 *Sf*9 cells into 25-cm^2 flasks containing 5 ml complete medium (*UNIT 16.8*). Prepare one flask for each putative recombinant plaque selected by method described in first basic protocol and one flask as noninfected control. Allow cells to attach ≥3 hr. Add 0.5 ml of 1-ml stock containing serum-free complete medium and recombinant plaque (step 8 first basic protocol) to each experimental flask. Reserve remaining 0.5 ml recombinant virus at 4°C for subsequent plaquings.
2. Incubate flasks 3 to 5 days at 27°C, monitoring daily for signs of infection. After 4 to 5 days, harvest cells by gently dislodging them from flask and transfer cells and culture medium to centrifuge tubes.
3. Centrifuge 10 min at 1000 × *g*, 4°C. If protein of interest is secreted, transfer culture supernatant to clean tube and proceed to step 6.
4. If protein of interest is intracellular, discard supernatant. Resuspend pellet gently in PBS to rinse cells, repeat centrifugation, and discard supernatant.
5. Boil cell pellets directly in 500 µl of 1× SDS/sample buffer and sonicate sample if it is too viscous due to presence of DNA. Continue until viscosity clears, then proceed to step 7. Alternatively, lyse cell pellets in 0.5 ml of an appropriate lysis buffer supplemented with protease inhibitors. Microcentrifuge 10 min at 4°C to clarify lysates and transfer supernatant to fresh tube. Remove 0.1 ml of each lysate and boil in 100 µl of 2× SDS/sample buffer. Freeze remaining lysate at –80°C.
6. Determine protein concentration in culture supernatant (step 2 for secreted proteins), or in the cell lysate (step 3 for intracellular proteins) using Bradford method (*UNIT 10.1*).
7. Analyze proteins in each sample by one of the following methods:
   a. Immunoblotting (*UNIT 10.8*); load 20 to 40 µg total cell protein/lane on an SDS-polyacrylamide gel (*UNIT 10.2*), including noninfected control.
   b. Coomassie blue staining of SDS-polyacrylamide gel (*UNIT 10.6*), loading 20 to 40 µg total cell protein/lane. Because recombinant virus is not pure, recombinant protein will be detected only if it is produced at very high levels in the infected cells.
   c. Functional assays, such as mobility shift DNA-binding assays, in vitro kinase assays, and nucleotide binding assays.
   d. Metabolic labeling (support protocol below).
8. Having identified which of the putative recombinant plaques is an actual recombinant (by one of methods suggested in step 7), plaque-purify it

so it is free from any contaminating wild-type virus (second basic protocol *UNIT 16.10*, omitting Xgal). This generally requires two to three rounds of plaque purification. Prepare large viral stock and titer recombinant virus (*UNIT 16.9*).

METABOLIC LABELING OF RECOMBINANT PROTEINS

SUPPORT PROTOCOL

Metabolic labeling in vivo is a sensitive way to detect recombinant proteins. However, when radiolabeling cells infected with a plaque (10,000 pfu), as opposed to a high-titer viral stock (10^8 pfu/ml), not all cells become infected simultaneously. Thus, [^{35}S]methionine labeling is performed later when infecting with a plaque (58 hr postinfections) at low MOIs than when infecting with a high-titer viral stock at high MOIs (35 to 40 hr postinfection).

Additional Materials

Methionine-free medium (special order from GIBCO/BRL or JR Scientific, or prepare from individual components as described by Summers and Smith, 1987)

0.1 to 0.5 mCi/ml [^{35}S]methionine (800 Ci/mmol; Du Pont NEN #NEG009A)

Variable-speed microcentrifuge (Fisher Scientific #59A)

1. Seed 2.5×10^6 cells into 60-mm tissue culture dishes containing 4 ml complete baculoviral medium. Prepare one dish to be infected with each putative recombinant plaque and one dish to be infected with a pure wild-type baculovirus plaque (step 8 of first basic protocol). Allow cells to attach 3 hr, then add 0.5 ml serum-free complete baculoviral medium containing plaques to each dish.

2. Place Parafilm around each dish and incubate at 27°C, monitoring daily for signs of infection. At ~58 hr postinfection, carefully remove culture medium, rinse cells once with methionine-free medium, and add 0.5 ml methionine-free medium with 0.1 to 0.5 mCi/ml of [^{35}S]methionine. Incubate 2 to 3 hr at 27°C.

3. Transfer cells and culture supernatant to centrifuge tube, centrifuge 10 min in tabletop centrifuge at $1000 \times g$, 4°C, and discard supernatant (for an intracellular protein). Resuspend cells in 1 ml PBS and transfer to microcentrifuge tubes. Microcentrifuge in variable-speed microcentrifuge at setting 3 for 20 sec. Discard supernatant.

4. Follow steps 5 and 6 in second basic protocol.

   If an antibody is available, it is recommended that the labeled lysates be immunoprecipitated prior to boiling in SDS/sample buffer and resolution by SDS-PAGE.

5. Resolve proteins in lysate by SDS-PAGE, loading 20 to 40 µg total cell protein or entire immunoprecipitate per lane. Visualize proteins by autoradiography. Inspect autoradiogram for protein of expected molecular weight that appears in cells infected with recombinant virus but not with wild-type baculovirus.

   Polyhedra are solubilized only under very alkaline conditions (0.1 M NaOH final). Less than 10% of polyhedra will be solubilized in SDS/sample buffer without prior disruption in alkali.

SUPPORT PROTOCOL

DETERMINING TIME COURSE OF MAXIMUM PROTEIN PRODUCTION

Expression of the recombinant protein does not occur until 15 to 24 hr postinfection (with accumulation until ~40 hr) because it is regulated by the polyhedrin promoter that is activated late in the lytic cycle of the virus. It is recommended that the time course of protein accumulation be charted for each protein because of their different stabilities in insect cells. The protein must be reasonably abundant for the method to be successful.

1. Seed 3×10^6 *Sf*9 cells into fifteen 60-mm tissue culture dishes containing 5 ml complete baculoviral medium. Allow cells to attach ≥3 hr, then infect seven dishes with wild-type virus and seven dishes with recombinant virus, each at an MOI of 10. Leave one dish noninfected.
2. Harvest cells at various times after infection (~15 to 72 hr postinfection) by transferring cells and culture supernatants to centrifuge tubes and centrifuging 10 min at $1000 \times g$, 4°C, in a tabletop centrifuge. Discard supernatant. Harvest noninfected cells 15 hr postinfection.
3. Resuspend cells in 1 ml PBS and transfer to microcentrifuge tube. Centrifuge as in step 2 of first support protocol (metabolic labeling). Follow steps 5 and 6 in second basic protocol (analysis of protein).
4. Analyze as in step 7 of second basic protocol. When staining with Coomassie blue (step 7b), look for a protein that appears as a function of time after infection with the recombinant virus but not with the wild-type virus.

SUPPORT PROTOCOL

MAXIMIZING INFECTION FOR RECOMBINANT PROTEIN PRODUCTION

1a. *For monolayer cultures*, seed one or more 150-cm^2 tissue culture dish with 1.8×10^7 cells/dish. Allow cells to attach firmly and remove culture medium. Add 5 ml serum-free complete baculoviral medium supplemented with recombinant virus at an MOI of 10; incubate 1 hr at 27°C. Remove viral inoculum and replace with 20 ml medium.

1b. *For suspension cultures*, grow cells in 100-ml spinner culture flask (*UNIT 16.9*) with 50 ml complete baculoviral medium to ~2×10^6 cells/ml. Centrifuge 10 min at $1000 \times g$. Resuspend cell pellet in 10 to 20 ml serum-free complete medium supplemented with recombinant virus at an MOI of 10. Incubate 1 hr at room temperature. Bring volume to 100 ml with complete medium and place cells in a 200-ml spinner flask or in two 100-ml spinner flasks. Incubate cultures at 27°C.

2. Harvest cells at time of optimum protein accumulation as determined in previous support protocol (~40 hr postinfection).
3. Process supernatant for secreted proteins or cells for intracellular proteins (second basic protocol).

Reference: Summers and Smith, 1987.

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