Purification and Transfection of Baculoviral DNA for Generating Recombinant Viruses

This unit describes how to obtain purified baculoviral DNA to be used in generating recombinant viral stocks. The plasmid pAC360 β-gal provides an easy colorometric assay for determining transfection efficiencies and recombination frequencies. It contains the *lacZ* gene fused in-frame with the polyhedrin gene and promoter, flanked on both sides by polyhedrin gene sequences. A homologous recombination event produces recombinant viruses that express a polyhedrin–β-galactosidase fusion protein. Recombinant viruses can be detected using medium containing the chromogenic dye Xgal because cells that produce the recombinant protein turn both the medium and the plaques blue.

BASIC PROTOCOL

16.10

PURIFICATION OF WILD-TYPE BACULOVIRAL DNA

Materials (see APPENDIX 1 for items with ✓)

Spodoptera frugiperda (*Sf*9) cells (ATCC #CRL 1711)
AcMNPV wild-type virus stock (*UNIT 16.9*)
0.1× TE buffer, pH 7.4
✓ Extraction buffer
10 mg/ml proteinase K, freshly prepared
10% *N*-lauroylsarcosine (sodium salt; filter sterilize and store at 4°C)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
100% and 70% ethanol
✓ 3.0 M sodium acetate

150-cm^2 flasks
50-ml conical and 15-ml polypropylene tubes
Beckman SW-27 or SW-28 (both precooled) and SW-41 rotors and tubes or equivalents

Infect cells with virus

1. Seed at least ten 150-cm^2 flasks with 1.8×10^7 *Sf*9 cells/flask. Allow cells to attach firmly, then infect with wild-type virus at MOI of 0.1 as in second basic protocol of *UNIT 16.9*. Incubate 3 to 5 days at 27°C. When occlusion bodies are observed in most cells, transfer viral supernatant (~200 ml) to four 50-ml conical tubes.
2. Centrifuge 10 min at 1000 × *g*, 4°C, and pour viral supernatant into four new tubes (sterile techniques are not required for remainder of this protocol). Repeat centrifugation to remove any remaining cells and place viral supernatant in SW-27 ultracentrifuge tubes.
3. Centrifuge 30 min at 100,000 × *g* (24,000 rpm in precooled SW-27 or SW-28 rotor), 4°C. Drain as much liquid from pellet as possible.

Separate virus from cellular contaminants

Examine viral pellet carefully. If pellet is pure (opaque, whitish appearance), proceed to step 7. In most instances, pellet will appear yellowish because of cellular debris. If this is the case, separate virus from cellular contaminants following one of the two methods listed below.

Purify viral pellet by sucrose-gradient fractionation:

4a. Resuspend viral pellet in 1 ml of 0.1× TE buffer, pH 7.4, by pipetting mixture up and down repeatedly. If it is difficult to resuspend viral pellet, let sit overnight at 4°C.

5a. Prepare two linear 25% to 56% sucrose gradients (ultrapure sucrose in 0.1× TE buffer, pH 7.4; filter sterilized and stored at 4°C) in SW-41 ultracentrifuge tubes (*UNIT 5.3*). Carefully layer 0.5 ml of viral suspension on top of each sucrose gradient. Centrifuge 90 min at 100,000 × *g* (28,000 rpm in SW-41 rotor), 4°C. Virus should be visible as a broad band in gradient.

6a. Remove viral band with Pasteur pipet and transfer to SW-41 ultracentrifuge tube. Dilute virus with 0.1× TE buffer, pH 7.4, to top of tube. Centrifuge 30 min at 100,000 × *g* (28,000 rpm in SW-41 rotor), 4°C. Drain as much liquid from pellet as possible. Proceed to step 7.

Purify viral pellet by microcentrifugation:

4b. Resuspend pellet in 3 ml extraction buffer by pipetting mixture repeatedly. If it is difficult to resuspend pellet, let sit overnight at 4°C.

5b. Transfer 1.5 ml of viral suspension into each of two microcentrifuge tubes. Microcentrifuge 5 min and transfer supernatants to 15-ml polypropylene centrifuge tube.

6b. Wash two pellets by resuspending each in 1 ml extraction buffer. Microcentrifuge 5 min and combine these two supernatants with previously pooled supernatants from step 5b in the 15-ml polypropylene tube. Bring volume to 9 ml with extraction buffer. Transfer 4.5 ml to two 15-ml polypropylene centrifuge tubes. Proceed to step 7.

Isolate DNA from purified virions

7. Resuspend virus pellet in 9 ml extraction buffer. Transfer 4.5 ml to two 15-ml polypropylene centrifuge tubes. Add 200 µg of 10 mg/ml proteinase K to each tube and incubate 1 to 2 hr at 50°C.

8. Add 0.5 ml of 10% *N*-lauroylsarcosine to each tube and incubate 2 hr or overnight at 50°C. Extract DNA twice with equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol (avoid shearing DNA).

9. Transfer aqueous phase containing DNA to another 15-ml tube using a wide-mouth (5- to 10-ml) pipet. Add 10 ml of 100% ethanol and mix gently by inverting tubes a few times. Place 10 min at –80°C.

10. Centrifuge 20 min in tabletop centrifuge at 2500 rpm, 4°C. Rinse DNA pellet with 70% ethanol and let air dry. Resuspend pellet in 800 µl TE buffer, pH 7.4. Transfer 400 µl to two microcentrifuge tubes and reprecipitate DNA by adding 40 µl of 3.0 M sodium acetate and 2 vol of 100% ethanol to each tube. Place 10 min at –80°C.

11. Microcentrifuge 10 min. Rinse DNA pellet with 70% ethanol and lyophilize. Resuspend DNA in 0.3 to 1.0 ml TE buffer, pH 7.4. Read A_{260} (*APPENDIX 3*) and calculate yield (~50 to 100 µg viral DNA). If difficulty is encountered resuspending DNA, heat ~15 min at 65°C. Store at 4°C for many months.

OPTIMIZATION OF PARAMETERS FOR BACULOVIRAL TRANSFECTION

SUPPORT PROTOCOL

Some viral DNA preparations are more pure than others. In most instances, 1 μg viral DNA is sufficient for transfection; however, because contaminating host DNA and RNA may result in higher absorbance readings than expected, it is often useful to optimize for the amount of wild-type viral DNA to use per transfection.

Additional Materials (see APPENDIX 1 for items with ✓)

Competent *E. coli* cells (strains HB101 or JM101; Table 1.4.5)
pAC360 β-gal vector (from M.D. Summers, *UNIT 16.8*, or Invitrogen)
LB plates containing 100 μg/ml ampicillin (*UNIT 1.1*)
Grace's Antheraea medium (GIBCO/BRL or JR Scientific) with 10% heat-inactivated (1 hr, 56°C) FBS and 50 μg/ml gentamycin
✓ Transfection buffer, room temperature
2.5 M $CaCl_2$ (tissue grade; filter sterilized; store at −20°C), room temperature
Complete baculoviral medium containing 150 μg/ml Xgal prepared in sterile DMSO
Agarose overlay containing 150 μg/ml Xgal prepared in sterile DMSO

1. Transform competent *E. coli* cells with pAC360 β-gal vector and select transformants on LB/ampicillin plates (*UNIT 1.8*). Pick one colony for large-scale plasmid preparation using alkaline lysis method and including a CsCl/EtBr equilibrium centrifugation (*UNIT 1.7*). Determine DNA concentration.
2. Seed seven 25-cm^2 flasks with 2.5×10^6 *Sf*9 cells/flask. Allow cells to attach for ≥3 hr. Remove medium and replace with 2 ml Grace's Antheraea medium/FBS/gentamycin (omit yeastolate and lactalbumin hydrolysate). Leave flasks at room temperature.
3. Set up seven sterile tubes containing either 0 μg, 0.5 μg, 1 μg, 2 μg, 4 μg, 6 μg, or 8 μg wild-type baculoviral DNA (from step 11 in first basic protocol). To each tube, add 2 μg pAC360 β-gal DNA and 950 μl transfection buffer, and mix.
4. Add 50 μl of 2.5 M $CaCl_2$ to DNA/transfection buffer mixture. Mix and aerate solution by bubbling air through with a 1-ml pipet. Incubate tubes 25 min at room temperature (to allow a precipitate to form).
5. Add solution containing the DNA precipitate to cells in 25-cm^2 flasks. Incubate flasks 4 hr at 27°C. Remove medium and rinse carefully with Grace's Antheraea medium/FBS/gentamycin (step 2 above).
6. Add complete medium containing 150 μg/ml Xgal. Incubate flasks 3 to 5 days at 27°C, observing daily for occlusion bodies as well as for a blue tint in medium (~day 3 or 4).
7. When cells are well infected (4 to 5 days), transfer transfection supernatant from each of seven flasks to sterile, conical centrifuge tubes. Centrifuge 10 min at 1000 × *g*, 4°C, in tabletop centrifuge. Transfer each viral supernatant to fresh, sterile tubes.

8. Prepare serial dilutions (*UNIT 1.11*) of transfection supernatants at 10^{-4}, 10^{-5}, and 10^{-6} (store remainder of viral stock at 4°C). Plaque virus (*UNIT 16.9*) using agarose overlays containing 150 μg/ml Xgal (seal plates with Parafilm).

9. Inspect plates daily for plaques that contain occlusion bodies (wild-type virus) and for those that are blue (recombinant virus). Plaques should be visible by 4 to 5 days postinfection. Count blue plaques and determine which concentration of wild-type baculoviral DNA resulted in highest frequency of recombinants. Use this concentration for future transfections with this stock of wild-type baculoviral DNA.

BASIC PROTOCOL

PURIFICATION OF RECOMBINANT BACULOVIRUS ENCODING β -GALACTOSIDASE

To become familiar with the morphological differences between plaques produced by wild-type virus versus those produced by recombinant virus, it is helpful to first practice plaquing β-gal recombinant virus alongside wild-type virus. This greatly facilitates screening recombinant viruses encoding the gene of interest.

Materials (see APPENDIX 1 for items with ✓)

Complete baculoviral medium *without* serum
Agarose overlay containing 150 μg/ml Xgal prepared in sterile DMSO
Spodoptera frugiperda (*Sf*9) cells (ATCC #CRL 1711)
✓ Complete baculoviral medium
1.5-ml screw-top cryostat tube

1. Using a sterile Pasteur pipet, pick a well-isolated, blue plaque (step 8 of first support protocol; ~10^4 pfu) and pipet agarose plug into sterile tube containing 1 ml serum-free complete medium. Vortex and prepare serial dilutions at 10^{-1}, 10^{-2}, and 10^{-3} in serum-free complete medium.

2. Plaque virus as in *UNIT 16.9*, using agarose overlays containing 150 μg/ml Xgal (to aid in finding recombinants). Repeat plaquing until pure stock of recombinant virus is obtained (generally two to three rounds).

3. Using a sterile pipet, pick a pure recombinant plaque (from step 2) and pipet agarose plug into a 25-cm^2 flask containing 2.5×10^6 *Sf*9 cells and 5 ml complete medium. Incubate 3 to 4 days at 27°C until majority of cells are infected. Virus titer should be between 5×10^7 and 1×10^8 pfu/ml (as determined by support protocol, *UNIT 16.9*).

4. Place 1 ml of this stock in a 1.5-ml screw-top cryostat tube at –80°C for long-term storage. Store remainder primary passage stock at 4°C. Grow large viral stock from primary passage stock and titer.

SUPPORT PROTOCOL

VISUAL SCREENING FOR RECOMBINANT BACULOVIRUSES

Screening for recombinant plaques is the most time-consuming and difficult aspect of the baculovirus expression system. It is critical that Lux tissue culture plates be used and that cells be plated to at least two different densities for plaquing. Plaque the pure wild-type baculovirus stock (*UNIT 16.9*) and the pure pAC360 β-gal recombinant virus stock (step 4 above) at dilutions of 10^{-7}, 10^{-8}, and 10^{-9} (*UNIT 16.9*). Do not include Xgal in the agarose overlay

as the blue color precludes a careful analysis of the plaque morphology produced by the β-gal recombinant virus. Plaques should be visible within 4 to 8 days. Visual screening for recombinant viruses can be done as described below. If screening for recombinant virus using these visual methods is unsuccessful, the plaque hybridization method can be used. This is similar to screening recombinant DNA libraries (Chapter 6; Summers and Smith, 1987).

Screening with a dissecting microscope. Invert plaquing dish on a black background beneath microscope and shine a bright light at an acute angle on plate. Scan cells infected with wild-type virus for plaques containing occlusion bodies. Plaques look very refractile, with a slight yellowish color. Scan cells infected with β-gal recombinant virus for plaques that lack occlusion bodies and appear grayish. Circle regions of the dish overlying the plaques. Examine circled plaques under an inverted light microscope at 400× to train your eye to the subtleties of each plaque morphology.

Screening with an inverted light microscope. Place plaquing dish right-side-up and scan for each type of plaque at 30× to 40×. Recombinant plaques have an orange tint, whereas wild-type plaques have a dark brown appearance due to the accumulation of occlusion bodies. While looking at the plaque under the microscope, place a dot on the region of dish underlying the plaque. Remove dish from microscope and circle plaque. Place dish back on stage of scope and view plaque at 400× magnification.

Direct visual screening. If plaques are well formed, it is sometimes possible to distinguish a wild-type plaque from a recombinant plaque by merely holding the dish up over your head and looking at the bottom of the dish directly. Regions surrounding wild-type plaques will look grayish-white whereas recombinant plaques will not.

Reference: Summers and Smith, 1987.

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