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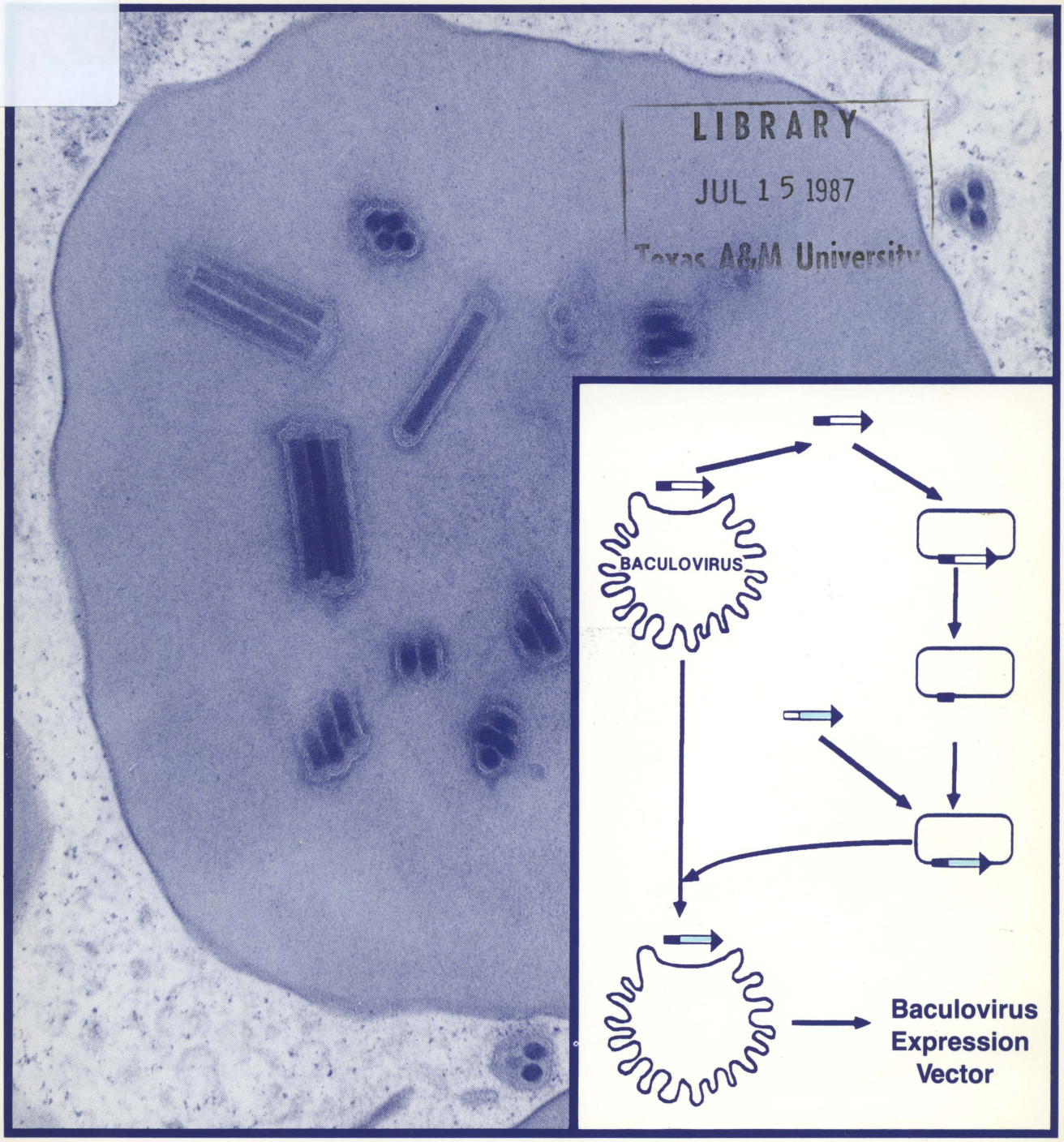

A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures

by Max D. Summers and Gale E. Smith

ON THE COVER:

The micrograph of the baculovirus depicted on the cover was kindly provided with the approval of Dr. C.Y. Kawanishi (Developmental Biology Division, EPA, Research Triangle Park, North Carolina), and is the nuclear polyhedrosis virus of *Heliothis armiger* (HaMNPV).

A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures

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The Texas A&M University System has a patent pending for the baculovirus expression vector process.

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Contents

1 Introduction

This guide is designed to aid researchers who are using the Baculovirus Expression Vector System (BEVS) for the expression of foreign genes (Figure 1). This is a helper-independent recombinant virus vector which has been used to express genes from many sources: bacteria, viruses, plants and mammals. Recombinant proteins have been produced as fusion or nonfusion proteins at levels ranging from 1–500 mg/liter [16] [25] [21] [22]. Because the factors which determine how well a foreign gene is expressed in this system are not yet well characterized, it is difficult to predict how efficiently different genes will be expressed. Many aspects of gene expression in this system are currently under study, and new vectors are being designed and evaluated for enhanced levels of expression.

The expression of the polyhedrin protein and its regulation are not well defined in molecular terms. It has been established that polyhedrin expression can be highly variable dependent upon the cell or tissue, virus and/or media components. Of major importance is the use of log phase Sf9 cells (a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE cells) that are at least 97% viable, a multiplicity of infection (MOI) of at least 5–10, and high quality medium and fetal bovine serum. An alteration in any of these factors can result in a significant reduction of the level of expression of any gene under polyhedrin control.

2 Baculoviruses: A brief overview

Autographa californica nuclear polyhedrosis virus (AcMNPV) is the prototype virus of the family *Baculoviridae* [15]. During AcMNPV infection, two forms of viral progeny are produced: extracellular virus particles (ECV), and occluded virus particles (OV) [27]. The latter are embedded in proteinaceous viral occlusions, called polyhedra (Figure 2) [18]. A polyhedrin protein with a molecular weight of 29,000 daltons is the major structural component of the viral occlusions [24]. In infected *Spodoptera frugiperda* cell cultures, polyhedrin accumulates to very high levels, routinely 1 mg/ml per 1.0–2.0 x 10^6 infected cells accounting for 50–75% of the total "stainable" protein of the cell detected on SDS-polyacrylamide gels.

The viral occlusions are an important part of the natural virus life cycle, providing the means for horizontal transmission of the virus. When infected larvae die, millions of polyhedra are left in the decomposing tissue. The viral occlusions protect the embedded virus particles from inactivation by environmental factors that would otherwise rapidly inactivate ECV. When larvae feed on contaminated plants, they ingest the polyhedra. The occlusions dissolve in the alkaline environment of the insect gut, releasing virus which invade and replicate in the cells of the midgut tissue. Secondary infection spreads to other insect tissues by the ECV form.

The cell biology of AcMNPV in *Spodoptera frugiperda* or *Trichoplusia ni* cells is summarized in Figure 2. Virus particles enter the cell by endocytosis or fusion and the viral DNA is uncoated in the nucleus. DNA replication occurs at about 6 hours post-infection (p.i.). At about 10 hours p.i. extracellular virus (ECV) is released from the cell by budding. Viral occlusions are detected by 24 hours p.i. Extracellular virus levels reach a maximum between 36 and 48 hours p.i. (an average of $8x10^6$ pfu/ml) but the polyhedrin protein continues to accumulate (greater than 4–5 days) until the infected cells lyse.

The polyhedrin gene of AcMNPV has been mapped (Figure 3) and sequenced (Figure 4). This gene has been shown to be nonessential for infection or replication of the virus [20]. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative viruses, which form plaques (Occ^-) that are distinctly different from those of wild-type, occlusion positive (Occ^+) viruses. These distinctive plaque morphologies provides a simple way to screen for recombinant viruses in which the wild type AcMNPV polyhedrin gene has been replaced with a hybrid gene of choice. The nonessential nature and high levels of expression of the polyhedrin gene and the ease which recombinant Occ^- viruses can be detected make the promoter of this gene particularly suitable for engineering as an expression vector.

3 Experimental outline

3.1 Selection of a transfer vector

The following outlines give step-by-step procedures currently used in our laboratory for constructing baculovirus expression vectors and producing recombinant proteins in infected insect cells. The details of each step are given in the Methods Section.

The first consideration is whether to fuse a foreign gene sequence before or after the polyhedrin translational start codon, ATG, where the A is nucleotide +1. In most cases it is desirable to express a mature protein instead of a fusion product; thus a transfer vector with a cloning site(s) upstream (in the 5' direction) from the polyhedrin translation initiator codon is required. The plasmids (transfer vectors) we have had the most experience with have one or more unique restriction sites following position -8, (The artificial BamHI linkers were inserted at point 8 bases before the first ATG and approximately 50 bases downstream from the polyhedrin transcriptional start site, Figures 5 and 6). These vectors can be used to insert a foreign gene sequence such that the first ATG is the foreign protein's initiator. The lengths of the 5' and 3' non-coding sequences of genes that we have expressed have varied from 3–400 bases [25]. We do not know the significance of these sequences with respect to their effect on gene expression, but we recommend that the length of 5' leader sequences of the foreign gene be shortened as much as possible. In general, the strategy has been

to insert the coding sequences of the foreign gene with as few noncoding flanking sequences as possible. All of the transfer vectors we have constructed have the polyhedrin polyadenylation signal intact. However, good levels of expression have been obtained using genes that have their own polyadenylation sites in addition to those of the polyhedrin gene.

In our laboratory, the transfer vector pAc373 (Figure 5) has been used for introducing many foreign genes into AcMNPV. This vector has a unique BamHI site following position -8. Good expression of nonfused proteins using pAc373 requires foreign genes that ideally have a short leader sequence containing suitable translation signals preceding an ATG start signal.

We also have a variety of transfer vectors for inserting open reading frames into the polyhedrin coding sequence. These vectors have unique BamHI or SmaI sites from position +5 to +175 (Figure 6) and are available upon request. We are currently comparing the efficiency of translation of recombinant proteins that have their own versus the AcMNPV polyhedrin initiator sequence. Preliminary evidence suggests that translation may be more efficient, and thus higher levels of expression are achieved, if synthesis of a recombinant protein begins at the polyhedrin translation initiator.

3.2 Production of a recombinant AcMNPV virus

After cloning a gene into an appropriate transfer vector, prepare at least 10 μg of highly purified plasmid DNA. *Spodoptera frugiperda* cells are sensitive to some contaminants found in crude plasmid preparations, which cannot be removed by phenol extraction or precipitation. The only consistently reliable method we have found for plasmid purification is CsCl-ethidium bromide gradient centrifugation. Impure preparations of plasmid DNA are toxic to the cells, and many cells may lyse shortly after transfection. This results in an apparently lower recombination frequency and increased difficulty in detecting recombinant viruses. To test the quality of a plasmid DNA preparation, simply include in all transfection experiments a control with AcMNPV DNA alone. At about 24 hours post-transfection, compare these cells to those transfected with AcMNPV DNA plus plasmid DNA. Sf9 cell viability should be greater than 97% for transfection experiments. Because the quality of the plasmid DNA is critical to the successful construction of recombinants, we have included a description of a large-scale plasmid purification protocol we routinely use. Because viral DNA quality (i.e., linear forms and extensive nicks should be minimized) also is important, a method for purification of AcMNPV DNA is also included.

To produce a recombinant virus, transfect *S. frugiperda* cells with a mixture of 1 μg AcMNPV viral DNA and 2 μg plasmid DNA. By the third or fourth day post-transfection, many (10–50%) of the cells should have viral occlusions visible in the nucleus and the virus (ECV) titer will be about 10^7 pfu/ml. The infected cells can best be observed with an inverted phase microscope at 250–400X magnification. If correct visualization of the Occ^-/Occ^+ phenotype is a

problem, first conduct these studies with a vector in which β-galactosidase is fused to the polyhedrin promoter. The visualization of a blue plaque (X-gal as the indicator) should help locate the Occ$^-$ plaque more quickly and with confidence.

After 3–4 days, plaques can be observed using a low power dissecting microscope. To visualize the plaques, place the inverted plates on a black background and illuminate with a strong side light plate at an acute angle. Plaques will first be visible after 2 or 3 days p.i. and will be fully formed by about 5 days p.i. Recombinant viruses can account for 0.1%–5% of the viral plaques. Purification of recombinants usually requires 2–3 rounds of plaque purification. At 3 or 4 days post-transfection, collect the medium, remove the cells by centrifugation and store the virus-containing supernatant at 4 $^\circ$ C. Serially dilute the virus into fresh medium to get 2 ml each of 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Inoculate duplicate plates with each dilution (1 ml/plate) and overlay with agarose and repeat the process of Occ$^-$ plaque selection.

3.3 Detection and purification of recombinant viruses

There are variety of ways to detect recombinant viruses, including visual screening of plaques for those formed from occlusion-negative viruses and filter hybridization of viral plaques. Although visual screening can be the most rapid approach, the ease with which this can be done depends on many factors and may be difficult at first without previous experience: access to a high quality dissecting microscope, optimal conditions for plaque-formation, relatively high levels of recombination, and some experience in discriminating between plaques formed from occlusion-negative and from occlusion-positive (wild-type) viruses. You may, therefore, prefer to detect recombinants by a plaque-hybridization procedure. The procedure is similar to colony hybridization. Although we have never tried using specific antibody probes by Western blotting technique to detect recombinant plaques, this should work equally well as reported informally to us by other scientists.

After identifying several recombinant viral plaques, pick these plaques from the agarose overlay with a 1000 μl pipet or sterile Pasteur pipet and transfer the agarose plug into 1 ml of medium. About 10,000 pfu can be recovered from a single plaque. Dilute the sample to 10–100 pfu/ml (10^{-1} to 10^{-3} dilutions) in medium. Inoculate duplicate plates with 1 ml of each dilution and then overlay with agarose. After 4–5 days the plaques will be visible. Usually a high percentage of the plaques will be formed from recombinant, occlusion-negative viruses. Examine the plaques with a dissecting microscope as described above and select plaques that appear less refractile. Using an inverted tissue culture phase microscope (250–400X magnification) examine these plaques more carefully. Start by looking at several plaques formed from the wild-type AcMNPV virus to become familiar with the appearance of viral occlusions in infected cells. Occlusion-negative (recombinant) plaques will not have any occlusions in the in-

fected cells, but the cells will be morphologically distinct from the surrounding uninfected cells. If visual examination still is inadequate for identification of recombinant plaque types, a second plaque hybridization may be done.

Once recombinant plaques have been identified, examine the plate under a dissecting scope and find a well-isolated plaque (at least several millimeters from a wild-type plaque). Pick the plaque with a 100–200 μl pipet and suspend it directly in 5 ml medium seeded with 1 x 10^6 cells in a 25 cm^2 flask. Within 3–4 days a majority of the cells should be infected with the recombinant virus, and the virus titer will be about 10^8 pfu/ml. Using an inverted phase microscope, check these cells very carefully for the presence of viral occlusions. If a significant number of cells have viral occlusions, plaque-purify again. We routinely store 1 ml of infected cell supernatant without the cells in a 1.5 ml screw-top cryostat tube at -80 ° C for long-term storage. We recommend that several aliquots of this stock be stored. The remaining 4 ml can be stored at 4 ° C and should be marked as passage 1 virus. We recommend that recombinant viral DNA be purified and analyzed by Southern hybridization anlysis to be certain that the foreign gene is in the viral genome and in the correct location. On occasion it is possible to get a significant number of false Occ$^-$ plaques. We do not understand the reason for this. It is therefore essential that you check the recombinant virus for the presence and location of the inserted genes. Sufficient viral DNA can be obtained for blot analysis by extracting total cell DNA from 5 ml of infected cells.

A few ml of virus are sufficient to conduct a number of experiments to determine whether the recombinant protein is being expressed. To prepare a large stock of virus, infect the desired volume of cells with the recombinant virus using a multiplicity of infection (MOI) of 0.1 to 1 pfu/cell. Because cells infected at this low MOI will continue to grow until all of them are infected, it is necessary to start with a relatively low cell density (1 x 10^6 cells/ml). For example, grow *S. frugiperda* cells in a 100 ml spinner flask with 50 ml medium to a density of 2 x 10^6 cells/ml. Pellet cells (1000 x g for 10 minutes) in a 50 ml conical centrifuge tube and then resuspend in 19 ml medium plus 1 ml 1st passage recombinant virus. After incubating at room temperature for 1 hour, add 80 ml medium and place in two 100 ml spinner flasks or one 250 ml spinner. Incubate cultures at 27 ° C for about 2 days. The titer of this second passage virus should be about 2–3 x 10^8 pfu/ml. Alternatively, seed 2 x 10^7 cells into a 150 cm^2 tissue culture flask and infect recombinant virus at a low MOI. We have used up to 6th passage virus (Ac373-β-interferon) with no detectable drop in yield of recombinant protein but have not yet done a systematic study of the effects of serial passage and MOI on the stability of a foreign gene in an AcMNPV expression vector. Although recombinant genes seem to be very stable in the baculovirus vectors we have studied, mutations in animal viruses generally can be minimized by limiting the number of serial passages and by infecting cells at a low multiplicity of infection.

3.4 Construction and selection of an AcMNPV-β-gal expression vector

To help you become familiar with making AcMNPV expression vectors, we have included the plasmid pAc360-β-gal (Figure 6). The advantage of using this transfer vector in your initial attempts at producing vectors is that the recombinant product, a polyhedrin-β-galactosidase fusion protein, can be detected with the chromogenic dye 5-bromo-4-chloryl-3-indolyl-β-D-galactopyranoside (X-gal). Prepare a (100X) stock of X-gal in DMSO at 20 mg/ml and store at -20 ° C. Transfect cells with a mixture of AcMNPV and pAc360-β-gal DNAs and incubate at 27 ° C for 3–4 days. Addition of X-gal to 150 μg/ml to the agarose overlay during plaque-purification will result in the production of dark blue plaques by the Ac360-β-gal recombinants. You may want to plaque-purify one of these, as it can be used to aid in the detection of recombinants in much the same way that the β-galactosidase gene in (pUC) plasmid vectors simplifies identification of recombinant plasmids in *E. coli*. If Ac360-β-gal viral DNA (Gal^+,Occ^- phenotype) is used as the viral DNA in transfections with transfer vector DNAs, recombinant viruses will be Gal^- and produce colorless plaques instead of blue ones. There are at least two technical difficulties that keep this approach from being the method of choice. First, the β-galactosidase diffuses from the cell and the blue color can obscure other recombinant and nonrecombinant plaques. Second, the mutation frequency may be somewhat higher with β-galactosidase (since the gene is much larger) than the polyhedrin gene, thus increasing the possibility of obtaining a mutant instead of a recombinant virus.

4 Methods

4.1 Insect cell culture

4.1.1 General handling techniques

- Fresh cell culture medium should be equilibrated to room temperature before use.
- When removing liquid from a flask of cells, the flask should be tilted at a 60 ° angle toward one bottom corner of the flask. Liquid should be allowed to flow to this corner, then removed very carefully with a pipet (avoid touching the cell monolayer).
- When removing liquid from a petri dish or other plate containing a cell monolayer, the plate should be tilted at a 45 ° angle so that liquid flows to one edge. Liquid should then be removed very carefully with a Pasteur pipet.
- When adding liquid to a flask, liquid should be gently pipetted down the side of the flask *opposite* the cell monolayer (that is, the top of the flask

when it is lying down for incubation). Avoid letting liquid flow down the monolayer — this may dislodge cells.

- When adding liquid to a plate, liquid should carefully be added dropwise to the center of the plate, allowing liquid to flow toward edges of the plate. One exception: agarose should be added to the edge of the plate very slowly, and allowed to flow to all edges of the plate.
- When resuspending cells in a flask or other vessel, cells should be gently dislodged from the growing surface by rapidly pipetting media across the monolayer. Avoid producing foam in resuspending the cells. Do not rap the small T-flasks to dislodge and resuspend the cells.
- When rocking flasks or plates (as for infecting with virus), each vessel should be rocked slowly four times by hand in each direction (north to south, east to west), watching carefully to be sure the liquid reaches all areas of the growing surface.
- To concentrate cells, gently dislodge and resuspend cells in fresh medium. Transfer cell suspension to a centrifuge tube of appropriate size (1.5 ml, 15 ml or 50 ml) and centrifuge for 10 minutes at 1000 x g. Certain insect cells are very sensitive to centrifugal force: the centrifugation conditions for each cell line need to be determined empirically. Carefully remove the supernatent without disturbing the pellet of cells. Add the desired volume of fresh media gently to the side of the tube and gently pipet to resuspend the cell pellet.
- When washing cells in a monolayer, gently add fresh media as described above. Avoid loss of cells by being *very* careful and *very* gentle. Rock fresh media across cell surface gently, then remove as described above.

4.1.2 Monolayer cultures

Spodoptera frugiperda (Sf9) (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852-1776; phone: 800-638-6597, Accession Number CRL1711) cells have a doubling time in TNM-FH media + 10% FBS of 18–24 hours and *should be subcultured three times a week.* Sf9 insect cells can be cultured at 27 ° C and will grow reasonably well at temperatures from 25–30 ° C. Carbon dioxide is not required and the cells can be maintained on the benchtop or in a hood as long as the temperature is maintained at a constant 27 ± 1.0 ° C. We *do not* routinely use antibiotics in the medium of our stock cultures. However, antibiotics are routinely used for larger volumes of cells to be used for experimental purposes. Subculturing in flasks can be done as follows:

1. Gently resuspend cells from a nearly confluent culture by rapidly pipetting the medium across the monolayer with a Pasteur pipet. Minimize foaming.

2. Transfer 0.5–1.0 ml of the culture (2–2.5 x 10^6 cells) to new 25 cm^2 flask containing 4 ml of fresh TNM-FH media plus supplements. Rock gently to wet the growth surface and distribute cells evenly.
3. Incubate at 27 ° C.

Note:

- Trypsin and other enzymes are not required for subculturing Sf9 cells.
- TNM-FH and Grace's medium do not contain pH indicators. The normal pH range for Sf9 cells in this medium is 6.2, and unlike mammalian cell cultures, the pH rises gradually as the cells grow, but usually does not exceed a pH of 6.4.
- As the cells divide, you may notice some which are either loosely attached or suspended in the medium ("floaters"). This is a normal occurrence and is often seen in older cultures and cultures which are "overgrown." If "floaters" constitute more than 5% of the culture, remove the old medium containing the "floaters" and replace with fresh medium before subculturing.
- It is desirable to seed cells in a serum-free medium to promote rapid and firm attachment for plaque assays, infections, etc. However, cells should not be left without serum for more than 2 hours.
- Theoretically, cells which take up trypan blue are considered non-viable. Cell viability can be checked by adding 0.1 ml of trypan blue (0.4% stock solution made up in buffered isotonic salt solution, pH 7.2–7.3) to 1 ml of cells and examining under a microscope at low magnification. Cell viability should be at least 97–98% for healthy log-phase cultures.
- Population doubling times for these cells will vary depending on growth conditions; as a general guide, however, healthy cultures in our laboratory double in 18–20 hours.
- When antibiotics are required, we recommend the use of 50 μg/ml gentamycin and 2.5 μg/ml amphotericin B ("Fungizone").

4.1.3 Suspension cultures

1. Determine the Sf9 ($\geq$97% viable) cell density using a hemocytometer.
2. Seed cells into spinner flasks to an initial density of about 0.5–1.0 x 10^6 cells/ml.
3. Incubate spinners at 27 ° C with constant stirring at 50–60 rpm.

4. For routine maintenance, subculture when the cell density reaches about 2–2.5 x 10^6 cells/ml, 2–3 times a week.

5. To subculture, remove 80% or more of the suspension and replace with an equal volume of fresh medium. Alternatively count cells ($\geq$97% viable) and re-seed in fresh medium at approximately 5 x 10^5 cells/ml.

6. At least once every 2 weeks, suspension cultures should be concentrated by gentle centrifugation, resuspended in fresh medium and transferred to clean, sterile spinner flasks. This will help prevent the accumulation of cell by-products and potential contaminants in the cultures.

7. Aeration (by diffusion, not by sparging) may be required in large ($\geq$ 100 ml) vessels for optimal growth of cells and production of virus. The optimal conditions have not been determined.

8. Sf9 cells are not anchorage dependent and may be transferred between monolayer and suspension cultures repeatedly without noticeable losses in viability or growth rate. Even after several months in suspension culture, the cells retain their ability to attach and grow in monolayer culture.

9. Suspension cultures are maintained in the same medium as monolayer cultures (TNM-FH + 10% FBS + antibiotics). No additional media components are required.

4.1.4 Freezing and storage of insect cell lines

Freezing cells:

1. Cells chosen for freezing should be from healthy, (97–98% viabilty) log-phase cultures.

2. Resuspend cells and then pellet by low speed centrifugation (1000 x g for 10 minutes). Resuspend in fresh medium to a density of at least 4 x 10^6 cells/ml.

3. Dilute cell suspension with an equal volume of freshly prepared freezing medium (complete medium plus 20% DMSO, filter sterilized) to give a final DMSO concentration of 10%. Keep cells on ice.

4. Dispense an appropriate volume (usually 1 ml/vial) of diluted cell suspension to freezing vials. Test the sealed vials for leaks.

5. Freeze cells slowly. Place vials in an insulated container, we use styrofoam racks, at -20 $^\circ$ C for 1 hour and then place at -80 $^\circ$ C overnight. Transfer the vials to liquid nitrogen storage on the following day.

Thawing cells:

1. Remove vial from liquid nitrogen and thaw rapidly with gentle agitation in a 37 ° C waterbath.
2. When the contents are almost thawed, quickly decontaminate the outside of the vial by immersing in 70% ethanol. Dry the vial and place it in an ice bath.
3. Transfer the cell suspension directly to a 25 cm^2 flask and dilute freezing medium with at least 5 volumes of cold (4 ° C) fresh cell culture medium.
4. Allow the cells to attach at room temperature (1 hour). Incubate at 27 ° C.
5. After the cells are well attached (2–3 hours later), replace the old medium with fresh medium.
6. Subculture the cells after they appear to have recovered fully. In some cases 3–4 days may be required before they are ready for routine subculturing.

4.1.5 Determining virus titer by end-point dilution

This procedure was originally described by Reed and Muench [17].

1. Make 10-fold dilutions of the virus to be titered in a final volume of 200 μl. Dilute with complete medium. Generally, 10^{-1} through 10^{-8} dilutions are prepared.
2. Add an equal volume of well suspended cell suspension to each dilution. For Sf9 cells, use 2.5 x 10^5 cells/ml. When titering virus containing the β-galactosidase gene, add X-gal to the cell suspension. This makes the scoring of the wells easier because of the formation of the indigo blue color in the positive wells. A 100X stock (20 mg/ml) of X-gal is prepared in DMSO, and stored frozen until ready to use.
3. Mix and suspend cell-virus preparation thoroughly (but gently).
4. Add 10 μl of each virus-cell mix dilution to each well of a 60 well plate (10 wells/dilution, so 6 dilutions will fit on a plate).
5. Add 0.5 ml (or less) sterile water to the edges of the plate to help prevent dehydration.
6. Place plates and a damp paper towel into a plastic bag and seal tightly. This keeps the plate and wells from drying out. Incubate at 27 ° C for 3-7 days, checking daily from day 3. If the virus is occlusion positive (produces polyhedra), score the results after 5 days. Let occlusion negative viruses incubate 5–6 days before scoring results.

7. Examine each well for infected cells. Any well that has any infected cells is scored positive. Total the number of positive wells and the number of negative wells for each dilution.

8. Calculate the virus titer by the Reed and Muench [17] method. A Fortran program is available to aid in this calculation. [2]

   The 50% end-point is determined by interpolation from the cumulative frequencies of positive and negative responses to occur in a dilution in which there would be 50% positive responses and 50% negative responses. The range of test dilutions should extend from the highest with all positive responses through the highest with all negative responses. Suppose that the data in the following table are the results obtained from titration of a virus using the above numerical values for scoring. The dilution in which there would be and expected 50% positive responses lies between the 10^{-6} and the 10^{-7} dilutions. The proportionate distance (PD) between these two dilutions is inferred by linear interpolation according to the formula:

   PD = ((% next above 50%) - 50%)/((% next above 50%) - (% next below 50%))

   In the example given, PD = 0.24 = (63.6 - 50.0)/(63.6 - 7.1).

Dilution	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Positive rate	10/10	6/10	1/10	0/10
Positive-number	10	6	1	0
Negative-number	0	4	10	10
Positive total	17	7	1	0
Negative total	0	4	13	23
Positive rate total	17/17	7/11	1/14	0/23
% Positive	100	63.6	7.1	0

   The following formula is used to calculate the 50% end-point:

   Log lower dilution (dilution in which position is next above 50%) = -6.0

   Minus the proportionate distance (PD) = -0.24

   Sum (50% end-point) = -6.24

   Log $TCID_{50}$ = -6.24

   $TCID_{50} = 10^{-6.24} = 1/(1.74 \times 10^6)$

[2] A Fortran program written by Dr. Verne A. Luckow is available to calculate the $TCID_{50}$ for determining virus titer by end-point dilution. The source code for this program can only be obtained over the world-wide BitNet communications network by sending a message to either (or both) of the following addresses: "LUCKOW@TAMENTO.BITNET" or "SUMMERS@TAMENTO.BITNET" if no response from first address. Sorry, requests for distribution on magnetic tapes or floppy disks cannot be honored.

This is the end-point dilution, the reciprocal of which is the titer in number of infective doses per unit of inoculum. If the inoculum was 0.005 ml per tube of cell culture the final expression would be:

$(1.74 \times 10^6)/0.005 = 3.48 \times 10^8$ $TCID_{50}$ per ml

9. Convert the $TCID_{50}$/ml to PFU/ml as follows:

   $TCID_{50}$/ml x 0.69 = PFU/ml

   $3.48\ 10^8 \times 0.69 = 2.4 \times 10^8$ PFU/ml

4.1.6 Determining virus titer by plaque assay

Titer the virus using this procedure adapted from Volkman and Summers [26] [27].

1. Prepare 10-fold dilutions from 10^{-1} to 10^{-9} in complete cell culture medium.
2. Set up duplicate plates for each dilution and do plaque assay as described earlier.
3. Count plaques. The optimal number is 50–100 per plate. Save the dilutions in case you don't achieve this range of plaques per plate (60 x 15 mm).
4. Save and store the original sample and all dilutions at 4 ° C until the results are verified.
5. Calculate the titer (PFU/ml) as follows:

   PFU/ml = 1/dilution x number of plaques x 1/(ml inoculum/plate).

   Example:

   1.24×10^7 PFU/ml = $1/10^{-4}$ x 124 plaques x 1/0.1 ml
6. You can also use the $TCID_{50}$ (end-point dilution) method to determine the titer of a virus stock. End-point dilution is cheaper, easier and more accurate.

4.1.7 Infection of cultured insect cells with virus

Monolayer cultures:

1. Count cells and seed into flasks or plates at an appropriate density (see chart 4.1.8). Allow cells to attach for 1 hour.
2. After cells are attached, remove medium and add the appropriate amount of virus (see footnote in suspension culture section below). Unless a specific multiplicity of infection (MOI) is desired, add the minimum volume of virus stock of inoculum required to cover the cells.

3. Incubate cells at 27 ° C for 1 hour.

4. Remove inoculum. Rinse with medium (if desired) and add fresh complete medium.

5. Incubate at 27 ° C for 2–4 days, visually examining cultures daily (see Volkman [27] for details).

6. To collect extracellular virus (for future inoculum, protein purification, etc.), transfer the infected cell medium to a centrifuge tube and pellet cells at 1000 x g for 15 minutes. Transfer supernatant to a fresh tube and store at 4 ° C. [3] The cell pellet may be saved for DNA or RNA purification, SDS-polyacrylamide gel electrophoresis of infected cell proteins, purification of non-secreted proteins, etc.

Suspension cultures:

1. Count cells and determine viability. For high virus titers and optimal expression of recombinant proteins, a cell viability of 97–98% or greater is recommended. Centrifuge the appropriate volume of suspension culture for the number of cells necessary for the experiment.

2. Calculate the amount of virus needed (ml of inoculum needed = MOI (PFU/cell) x number of cells/titer of virus (PFU/ml)). [4]

3. Resuspend the pelleted cells in the calculated amount of virus inoculum. Add complete medium to an initial density of approximately 1 x 10^7 cells/ml.

4. Incubate at 27 ° C or room temperature for 1 hour. Agitation is not necessary.

5. Resuspend the cells by adding fresh medium to an appropriate density (usually between 1 x 10^6 and 5 x 10^6 cells/ml). Transfer the suspension to a spinner flask. Also transfer 5 ml of the cell/virus suspension to a 25 cm^2 flask to monitor the infection process.

[3] We do not recommend the storage of routinely used virus stocks at temperatures below 4 ° C. Our data suggests that repeated freezing and thawing of stocks of AcMNPV and its derivatives results in substantial decreases in virus titer. In contrast, viruses stored at 4 ° C for periods in excess of 2 years show virtually no change in titer. We do, however, recommend the storage of small aliquots of important isolates at -80 ° C. These should be used as very long term storage reserves only.

[4] For most work, an MOI of 10 or greater will give a fairly synchronous infection and produce high ECV titers, recombinant protein levels, etc. It is not known if defective interfering particles are produced in cells infected with AcMNPV at a high MOI as is the case for many other viruses. Although we have never detected a problem with a build up of defective interfering particles, it is advisable to produce viral inoculum from relatively low passage numbers (preferably 5 or less) and infect cells at an MOI of less than 1. Occasional re-purification from individual plaques will assure that low passage virus inoculum is always available.

6. Incubate the culture at 27 ° C with constant stirring for 2–4 days. Check the progress of the infection regularly by making wet mounts of the infected cell suspension and examining cells with a phase microscope.

7. To collect culture medium (for future inoculum or protein purification), pellet cells by centrifugation and transfer supernatant to a sterile bottle. Store at 4 ° C.

4.1.8 Seeding densities

The chart below gives approximate seeding densities for typical vessel sizes. Infection at these densities will usually give high virus titers ($\geq$1 x 10^8 PFU/ml); however, for maximum levels of recombinant proteins, higher densities ($\geq$3 x 10^6 cells/ml) may be desirable.

Type of Vessel	Cell Density	Minimum Virus Volume	Incubate in Final Volume
96 well plate	2.0 x 10^4/well	10 μl	100 μl
24 well plate	3.0 x 10^5/well	200 μl	500 μl
60 mm^2 dish	2.5 x 10^6/dish	1 ml	3 ml
25 cm^2 flask	3.0 x 10^6/flask	1 ml	5 ml
75 cm^2 flask	9.0 x 10^6/flask	2 ml	10 ml
150 cm^2 flask	1.8 x 10^7/flask	4 ml	20 ml
spinners (all)	1.5–2.0 x 10^6/ml	(based on MOI)	(based on size)

4.2 Cloning genes into AcMNPV transfer vectors

4.2.1 Restriction digest of AcMNPV transfer vectors

This procedure is based on that described by Maniatis, et al. [14].

1. To digest 10 μg of pAc373, combine the following in a 1.5 ml microfuge tube:

   10 μg plasmid DNA

   25 μl 10X restriction enzyme buffer

   20 units enzyme

   sterile distilled water to bring volume to 250 μl

2. Incubate at 37 ° C for at least 3 hours.

3. Check about 0.5 μg (12.5 μl) on an agarose gel to be sure the digestion is complete. If not completely digested, add an additional 10 units of enzyme and incubate for 3 more hours.

4. After the plasmid is well digested, dephosphorylate by adding 1 unit Calf Intestinal Alkaline Phosphatase (CAP) per μg DNA. Add the enzyme directly to the restriction enzyme digest and incubate 30 minutes at 37 ° C (for 5' extensions) or 50 ° C (for blunt ends and 3' extensions).

5. Inactivate CAP by adding EDTA to 25 mM and SDS to 0.5% and incubate at 65 ° C for 15 minutes.

6. Extract with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Add 125 μl of 7.5 M ammonium acetate and 800 μl ethanol, and precipitate at -80 ° C for 10 minutes.

7. Pellet DNA in a microfuge for 10 minutes.

8. Rinse the pellets with cold 90% ethanol, remove all ethanol, spin dry to remove excess ethanol and resuspend in 50 μl (200 ng DNA/μl) of 0.1X TE (1X TE = 10 mM Tris-HCl, 1mM EDTA, pH 7.6) buffer. Store at -80 ° C.

4.2.2 DNA ligations

This procedure is based on that described by Maniatis, et al. [14].

It is usually necessary to purify foreign gene fragments before ligating them to pAc373 or similar vectors. The purified fragment should be checked on a gel to be certain that it is not degraded or contaminated with its vector DNA. A 1–3 μl sample of the purified fragment should give a clearly visible band on the gel, $\geq$50 ng.

Set up the ligation to linearized, CAP-treated pAc373.

pAc373 (200 ng)	1.0 μl
purified insert (1–5X molar excess of insert to pAc373)	1–3 μl
H_2O	6.5–4.5 μl
10X ligation buffer	1 μl
T4 DNA ligase (1–20 units/μl)	0.5 μl
Total volume	10 μl

Incubate at 14 ° C for 3 hours or overnight.

- The more gel-purified fragment that is required, the greater the possible concentration of any contaminants that might inhibit the ligation reaction; therefore, keep the volume of gel-purified fragment in the ligation mix to an absolute minimum.

- Use of additional ligase (0.5 to 1 unit of ligase is a vast excess) may result in glycerol concentrations reaching inhibitory levels.

4.2.3 Transformation of bacterial strains

We routinely use frozen competent cells [6]. Because these cells are sensitive to pH, it is necessary to neutralize the ligation mix before adding the DNA to the cells.

1. To neutralize the reaction, add 1.5 μl 1 M Na-(2[N-morpholino]ethane sulfate]) (MES) buffer, pH 6.25 to each 10 μl ligation reaction before adding it to 200 μl competent cells.
2. Incubate competent cells mixed with ligation reaction mix on ice for 60 minutes.
3. Incubate cell mix at 42 ° C for 2 minutes.
4. Add 800 μl SOC medium (2% Bacto Tryptone, 2% Bacto Yeast Extract, 10 mM NaCl, 10 mM $MgSO_4$, 20 mM glucose) and incubate at 37 ° C for 60 minutes.
5. Spread 50 to 500 μl on selective media plates. Incubate plates at 37 ° C overnight.
6. Screen possible recombinant colonies by isolation and restriction enzyme analysis of plasmid DNAs. Analysis of 12 colonies is usually sufficient to identify a clone containing a plasmid with the insert introduced into pAc373 in the correct orientation relative to the polyhedrin promoter.

4.3 Plasmid DNA isolation

4.3.1 Minipreps

This miniprep procedure is based on that described by Holmes and Quigley [9].

1. Using a sterile stick or loop, inoculate 2–4 ml of Luria Broth (LB) (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl) + the appropriate antibiotic with a single colony from a freshly prepared plate.
2. Incubate cultures at 37 ° C for 12–18 hours.
3. After incubation, centrifuge cultures at 2500 rpm for 10 minutes.
4. Remove the supernatant from each tube by aspiration (we use a Pasteur pipet and tubing attached to a water faucet aspirator). Be sure to remove all the liquid, but do not disturb the pellet.
5. Resuspend each cell pellet in 500 μl STET buffer and transfer the suspension to a 1.5 ml microcentrifuge tube.

   STET Buffer (for plasmid DNA preps)

80.0 g Sucrose

50 ml Triton-X-100

7.72 g $Na_2EDTA{\cdot}2H_2O$

6.05 g Tris

Add to distilled water and mix to dissolve. Adjust pH to 8.0, bring volume to 1 liter, and sterilize by autoclaving (optional). Store at 4 ° C or room temperature. Warm to room temperature before using.

6. Add 50 μl of 10 mg/ml lysozyme solution (prepared fresh in STET buffer) to each tube and vortex briefly.
7. Incubate samples for 2–5 minutes at room temperature.
8. Place the tubes in a boiling waterbath for 45 seconds, then cool in an ice bath for 2 minutes or longer.
9. Microfuge samples for 10 minutes.
10. Carefully remove the gelatinous cell pellet from each tube using sterile pipet tips.
11. Add 1 ml of ethanol to the supernatant left in each tube and vortex thoroughly.
12. Precipitate at -80 ° C for 10 minutes (or -20 ° C for 2 hours or longer) and microfuge for 10 minutes.
13. Remove the ethanol solution from each tube using a Pasteur pipet or aspirator.
14. Fill each tube with cold 90% ethanol (-20 ° C) and then remove ethanol again (optional).
15. Microfuge samples for about 10 seconds to "spin dry" and remove remaining ethanol with 200 μl pipet.
16. Add 100 μl of 0.1X TE to each sample and incubate at 65 ° C for at least 10 minutes.
17. Tap or vortex tubes to resuspend the DNA/RNA/protein pellet.
18. Store samples at or below -20 ° C, and heat to 65 ° C for 10–20 minutes before each use.

Restriction digests:

The amount of miniprep DNA that is needed for restriction enzyme analysis will vary, but the following are guidelines to help you determine the minimum

needed and thus, the minimum amount of restriction enzyme required for complete digestion. If the fragments you are trying to visualize are at least 500 base pairs (bp), about 0.5–0.75 μg of plasmid DNA (5–10% of a typical miniprep) per digest will be more than adequate. About 1.0 μg of DNA is needed to see fragments less than 500 bp. Because you will usually have 12 or more minipreps to analyze at a time, it is often convenient to make a "master mix" solution containing the buffer, enzyme, and water needed to add to each sample. For example, to digest 24 minipreps with both SmaI and SalI (including a little extra to allow for pipetting losses) prepare the following:

First enzyme digest (SmaI):

Component	One Sample	25 Samples
10X SmaI salts	5 μl	125 μl
distilled water	40 μl	1000 μl
SmaI (10 units/μl)	0.4 μl	10 μl
Total volume	45 μl	1125 μl

1. Add the buffer, water, and enzyme for all the samples to a 1.5 ml microfuge tube (this is your "Master Mix").
2. Add 5 μl of each miniprep DNA (enough for 2 digests) to a fresh 1.5 ml microfuge tube.
3. Add 45 μl of Master Mix to each miniprep DNA tube, vortex at low speed to mix, and incubate 3 hours at the recommended temperature (37 ° C).
4. Using a round-bottom 96 well plate, add about 5 μl STOP solution to each of 24 wells.
5. Transfer 20 μl from each DNA digest to the 96 well plate; mix with STOP solution and then apply to agarose gel. (Note that using the multi-well plate is rapid, saves on tubes, and allows you to use only a single pipet tip for each sample).
6. Store the remaining 30 μl of each digest at -20 ° C until the results of the gel are known; if any samples are not fully digested, incubate those samples further before continuing.

Second enzyme digest (SalI):

Component	One Sample	25 Samples
10X High Salt	3.0 μl	75 μl
SalI (10 units/μl)	0.2 μl	5 μl
Total volume	3.3 μl	75 μl

1. Heat the SmaI-digested DNAs for 10–20 minutes at 65 ° C.
2. Add 3 μl of the second Master Mix to each sample, vortex at low speed to mix, and incubate 3 more hours at 37 ° C.
3. Apply to gel as described above.

Notes:

- This method can also be used for preparing minipreps from pBR322 and M13 samples. For these (which yield less DNA), use four times the amount of miniprep DNA as listed above, but digest with the same amount of enzyme (2 units per sample).
- If you need to digest large amounts of miniprep DNA scale-up the digestion components proportionately. As a general policy, use no more than 25 μl DNA in a 100 μl final reaction volume. Adding more DNA will change the proportions and may result in poor digestion (the contaminants present in minipreps will interfere with restriction enzymes unless diluted).
- Timing is critical when doing minipreps; work quickly, and do not stop for long at any point in the procedure until the samples are in ethanol. Nucleases present in the bacterial cells will degrade your DNA very rapidly. Because there is no phenol extraction step, these nucleases are never removed. This will not be a problem as long as they are inactivated by heating the miniprep DNAs to 65 ° C before each use.

4.3.2 Maxipreps

This maxiprep procedure is based on that described by Holmes and Quigley [9].

1. Using a sterile stick or loop, inoculate 1 ml of LB supplemented with an appropriate antibiotic with a single colony of bacterial cells from a freshly prepared plate.
2. Incubate at 37 ° C for 12 to 18 hours.
3. Transfer the 1 ml culture to 200 ml LB + antibiotics in a 500–1000 ml flask and incubate in a shaking incubator (37 ° C) overnight (12–18 h).
4. Transfer the 200 ml cultures to centrifuge bottles, and centrifuge at 2500 rpm for 10 minutes.
5. Pour off the supernatant and then remove any remaining medium from the bottles by aspiration.
6. Resuspend each cell pellet in 30 ml STET buffer (room temperature) and transfer to a 125 ml flask.

7. Add 3 ml of 10 mg/ml lysozyme (prepared fresh in STET buffer) and swirl each flask to mix.
8. Incubate about 5 minutes at room temperature.
9. Gently swirl (about once every 5 seconds) each flask directly over a flame. The cells will begin to coagulate and turn white. Watch the bottom of the flask as you swirl it; the moment you see bubbles forming transfer the flask to a boiling water bath for 45 seconds.
10. Cool the flasks in an ice water bath for 2 minutes or longer, then slowly pour the viscous mixtures into 50 ml round bottom centrifuge tubes.
11. Centrifuge for 15 minutes at 16,000 rpm in a preparative centrifuge.
12. Carefully pour the supernatant from each tube into a 50 ml disposable polypropylene centrifuge tube. The cell pellet should be flocculent and occupy about one fourth to one half of the tube; if a hard pellet has formed, you may have overheated the cells. Do not panic. Reasonable yields of DNA can often still be obtained.

At this point choose from either of two pathways to concentrate your DNA.

Ethanol precipitation of DNA:

1. Add either 1 volume of isopropanol or 2 volumes of 100% ethanol and mix well.
2. Precipitate at -80 ° C for 20 minutes (or -20 ° C for 2 hours or longer). Then centrifuge at 2500 x g for $\geq$15 minutes.
3. Pour off the ethanol solution and invert the tubes on a paper towel to drain off the excess alcohol.
4. Add about 10 ml of cold (-20 ° C) 90% ethanol to each tube (rinsing the sides of the tube as you add it). Then pour off and again allow the excess to drain onto a paper towel.
5. Add 2.5 ml Extraction Buffer to the pellet and vortex the lysate just enough to loosen it from the bottom of the tube.

   Extraction Buffer (for DNA purification)

   12.1 g Tris

   33.6 g $Na_2EDTA{\cdot}2H_2O$

   14.9 g KCl

   Dissolve in distilled water, adjust pH to 7.5, and bring volume to 1 liter. Filter sterilize and store at 4 ° C or room temperature.

6. Add 100 μl of 10 mg/ml of RNaseA (dissolved in 0.1X TE and pre-treated by boiling for 10 minutes) and incubate for 30 minutes at 37 ° C. Add approximately 200 μg proteinase K to each tube and then incubate at 40–50 ° C for about 30 minutes. If the pellets are not completely broken up after 30 minutes, vortex briefly or mix gently with a Pasteur pipet.

7. Add 250 μl of 10% Sarcosyl (DO NOT SUBSTITUTE SDS) and continue incubation for 3 hours or longer (overnight, if more convenient).

Polyethylene glycol precipitation of DNA:
This procedure is similar to that described by Humphreys, et al.[11].

1. Add 100 μl of 10 mg/ml of RNAseA (dissolved in 0.1X TE and pre-treated by boiling for 10 minutes) to the cleared lysate and incubate for 30 minutes at 37 ° C.

2. Add NaCl to 0.5 M and solid PEG-8000 to 10% (wt/vol) (e.g., 2 ml of 5 M NaCl and 2 g PEG to 20 ml of lysate). Vortex gently to dissolve PEG. Incubate on ice for 2 hours or overnight if convenient.

3. Collect the PEG/DNA pellet by low speed centrifugation. Discard the supernatent.

4. Resuspend the pellet in $\geq$3 ml 0.1X TE.

Preparation of CsCl gradients:

1. Add 100 μl of 5 mg/ml ethidium bromide per ml of DNA solution to each tube.

2. Add 1.02 g of CsCl per ml of DNA solution to each tube and gently vortex to dissolve.

3. Pour solution into small ultracentrifuge tubes and overlay each tube with mineral oil or paraffin oil.

4. Centrifuge the gradients to equilibrium in an appropriate rotor (45K rpm for $\geq$15 hours in a 70.1 Ti fixed angle rotor or 32K for 36–48 hours in an SW60 swinging bucket rotor).

5. Remove gradients carefully from rotor. If the yield is good, there should be two well-separated bands of DNA visible (without the aid of a longwave UV lamp).

6. If you are using reusable polycarbonate centrifuge tubes, use a 1000 μl pipetter and carefully remove the CsCl oil above the two DNA bands. With a clean pipet tip, remove just the upper band (linear and nicked DNA). With another clean pipet tip, collect the lower band (covalently

closed circular DNA) and put it into a 15 ml polypropylene centrifuge tube. If you are using disposable polyallomer tubes, puncture the top of the tube with a 22 gauge needle. Puncture a second hole at the bottom and collect the plasmid band in a tube as it slowly drips out of the bottom.

7. Add water to bring the volume in each tube to about 6 ml. Extract the ethidium bromide from the DNA using an equal volume of isoamyl alcohol.

8. Remove the pink (upper) phase and discard it. Add more isoamyl alcohol and repeat the extraction until the upper phase is colorless. The number of extractions that are required will vary, but usually three are sufficient.

9. After the final extraction, the bottom (DNA) phase should be clear and colorless. Remove the last traces of isoamyl alcohol with a pipetter.

10. There should be about 5 ml of DNA solution left in each tube (if not, add water to 5 ml). Add 2 volumes of absolute ethanol to each tube and mix thoroughly. If the CsCl concentration is too high, the CsCl will precipitate when ethanol is added; the addition of water should prevent this. If the CsCl does precipitate, transfer half of the mixture to another 15 ml centrifuge tube, add 2.5 ml water to each tube, mix until the CsCl dissolves, and then add an additional 5 ml ethanol to each tube.

11. Precipitate at -20 ° C overnight or -80 ° C for 10 minutes. Pellet at 2500 x g for 20 minutes.

12. Pour off the ethanol and invert tubes for a few minutes on a paper towel. Rinse with 5 ml of cold 90% ethanol. Let the tubes drain for a few minutes.

13. Centrifuge for 1 minute and remove the remaining alcohol with a pipet.

14. Add 500 μl of 0.1X TE to each pellet and resuspend at 65 ° C for 10 minutes or longer.

15. Store the DNA at 4 ° C. DNA prepared by this method should be stable for years when stored at 4 ° C. Yields should be about 500–2000 μg of DNA with pUC plasmid clones and about 100–500 μg with pBR and M13 clones per 200 ml of bacterial cells.

4.4 Transferring genes into the AcMNPV genome

4.4.1 Transfection of Sf9 cells (Method I)

Plasmids containing foreign genes are transferred to the AcMNPV genome by recombination *in vivo*, using a modification of the calcium phospate precipitation technique [5] as modified for insect cells [1] [2].

1. Seed Sf9 cells into 25 cm^2 flasks at a density of 2.0 x 10^6 cells per flask. Allow cells to attach for at least one hour.

2. Place 1 μg of AcMNPV DNA in a 1.5 ml polypropylene tube. Add 2 μg of plasmid DNA (containing your foreign gene) to the same tube.

3. Remove media from flasks and replace with 0.75 ml of Grace's medium + 10% FBS + antibiotics. Leave flasks at room temperature.

4. Add 0.75 ml of Transfection Buffer (25 mM HEPES, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$) to the DNA in the tube and vortex.

5. Add the DNA solution dropwise to the Grace's medium already in the cell culture flasks. Calcium phosphate precipitates form due to the $CaCl_2$ in the transfection buffer and the phosphate in the medium.

6. Incubate flasks at 27 ° C for 4 hours.

7. Remove the medium from each flask with a Pasteur pipet. Rinse flasks carefully with fresh TNM-FH + 10% FBS + antibiotics, and add 5 ml of TNM-FH + 10% FBS + antibiotics.

8. Incubate 4–6 days, checking cells with an inverted microscope for signs of infection [27]. Positive signs of infection may include:

   (a) Appearance of polyhedra.

   (b) As much as a 25–50% increase in diameter of cells, but more easily discerned is nuclear expansion which is characteristic of cytopathic effects (CPE).

   (c) Cell lysis late in infection (after 4-5 days).

9. When the infection is at an advanced stage, plate the virus (ECV) on fresh monolayers and identify recombinant viruses by plaque hybridization and purify by plaque assay.

4.4.2 Transfection of Sf9 cells (Method II)

1. Seed Sf9 cells into 25 cm^2 flasks at a density of 2.5 x 10^6 cells per flask. Allow cells to attach. Meanwhile, warm HEBS/CT and $CaCl_2$ solutions to room temperature.

2. Remove media from flasks and replace with 2 ml of Grace's medium + 10% FBS + antibiotics. Leave flasks at room temperature.

3. Mix 1 μg of AcMNPV-E2 DNA and 2 μg of pAc recombinant plasmid DNA in a tube.

4. Add 950 μl of HEBS/CT solution to the DNA tube and vortex.

   HEBS/CT (for transfections)

   Prepare 100 ml of 10X HEBS stock as follows:

1.37 M NaCl	8.0 g
0.06 M D(+) glucose	1.081 g
0.05 M KCl	0.373 g
0.007 M $Na_2HPO_4 \cdot 7H_2O$	0.188 g
0.2 M HEPES	4.766 g

   Dissolve all of the above in about 80 ml of distilled water. Adjust to pH 7.1 with fresh 5 M NaOH. Bring volume to 100 ml. Filter sterilize and store at -20 ° C.

   To prepare 100 ml of 1X HEBS/CT, combine 10 ml of 10X HEBS stock, 1.5 ml of sonicated calf thymus (CT) DNA (1 mg/ml) and 88.5 ml sterile distilled water. Adjust to pH 7.05–7.10 and refilter if necessary. Store at 4 ° C. The pH is very critical. Therefore, check it before each use, adjust it if necessary. If the pH is below 7.0 the DNA will not form a visible precipitate and the transfection will be very inefficient. If the pH is too high, a precipitate with very large particles will form and many of the cells will die. At the correct pH, a very fine precipitate will form over the cells that will give them a "milky" appearance and the particles will be just visible at 400X magnification.

   2.5 M $CaCl_2$ (for transfections)

   Dissolve 9.181 g of $CaCl_2 \cdot 2H_2O$ in 20 ml of distilled water. Bring the volume to 25 ml and filter sterilize. Dispense in 0.5 ml aliquots and store at -20 ° C. Use a fresh tube for each set of transfections; do not re-freeze.

5. Add 50 μl of 2.5 M $CaCl_2$ to the DNA/HEBS/CT mixture and vortex again. Incubate at room temperature for 30 minutes.

6. Add the DNA/HEBS/CT solution to the Grace's medium already in the cell culture flasks.

7. Incubate flasks at 27 ° C for 4 hours.

8. Using a Pasteur pipet, remove the medium from flasks. After rinsing flasks carefully with fresh Grace's medium + 10% FBS + antibiotics, add 5 ml of TNM-FH + 10% FBS + antibiotics.

9. Incubate at 27 ° C for 4–6 days, checking cells with an inverted microscope for signs of infection (described in the previous section).

10. When the infection is at an advanced stage, plate the virus (ECV) on fresh monolayers and identify recombinant viruses by plaque hybridization and purify by plaque assay.

4.4.3 Plaque assay

1. Seed Sf9 cells (97–98% viable) into Lux 60 x 15 mm culture plates [5] at a density of 2.0 x 10^6 viable cells per plate in TNM-FH medium. [6] Rock plates to distribute cells evenly, and allow cells to attach for at least 1 hour. Examine the cells from several plates under an inverted microscope to confirm cell attachment. They should not be confluent since they will grow and divide over the course of the plaque assay.

2. Prepare 10-fold dilutions of virus inoculum in the range of the expected titer allowing approximately 1 ml of diluted virus for each plate. Dilute transfection mixes 10^{-3} through 10^{-5}, supernatents from dot blot experiments 10^{-2} through 10^{-4}, and viruses picked from a plaque 10^{-1} and 10^{-2}. Save 1 ml of the least diluted stocks in sterile Eppendorf tube and store at 4 ° C until you have isolated the recombinant virus and confirmed its structure by DNA hybridization and/or restriction enzyme analysis.

3. After cells have attached, remove media and add 1 ml diluted virus inoculum to each plate. Rock plates to distribute virus evenly.

4. Incubate plates for 1 hour at room temperature or 27 ° C.

5. While plates are incubating, prepare 1.5% low melting point (SeaPlaque) agarose overlay as follows:

   (a) Calculate the amount of overlay needed (4 ml per plate).

   (b) For each 100 ml of overlay (final volume), resuspend 1.5 g of agarose in 50 ml H_2O in a small bottle. Autoclave it for 15–20 minutes.

   (c) For each 100 ml of overlay, combine in another bottle 40 ml 2X TNM-FH, 10 ml fetal bovine serum and sufficient antibiotic stocks for the desired final drug concentrations.

   (d) Equilibrate both solutions to 37–40 ° C in a waterbath, and mix them together in one bottle. Leave the overlay in the waterbath until ready to use.

6. At the end of the 1 hour incubation period, use Pasteur pipets to remove all inoculum from each plate. Slowly add 4 ml of overlay to the edge of each plate. Rock each plate immediately after adding overlay to spread agarose evenly.

7. Leave plates undisturbed for at least 1 hour to allow overlay to solidify.

[5] We tested many, but not all brands of culture dishes, and found that Lux Contour Dishes (Miles Laboratories, Inc.) were the most suitable for producing uniform monolayers on which plaques are clearly visible and distinguishable from the background of uninfected cells.

[6] For faster and more efficient attachment, resuspend cells in serum-free medium before seeding.

8. Incubate plates in a humid environment for 4–6 days, or until plaques are well-formed. If a 27 ° C incubator with high (80–100%) humidity is not available, seal the plates in a plastic bag or sealed container and add damp paper towels to provide humidity.

9. Three rounds of plaque purification are usually sufficient to isolate recombinant viruses. If you use dot blot hybridization to identify recombinants initially, you may need to carry out an extra round of plaque purification, but it is well worth the time invested.

4.4.4 Visual screening for recombinant viruses

1. When plaques are well-formed (4–6 days p.i.), examine plates using a dissecting microscope. Use the highest quality dissecting microscope that is available. Place the lamp such that the bottom of the inverted plate is illuminated at an acute angle and with a very intense light source (e.g., an old slide projector). Against a black (velvet) background the wild-type plaques should look very refractile, almost crystal-like, with a slight yellow color.

2. Look at the plates which have been infected with a 10^{-4} dilution of virus first. At this dilution the plate should be covered with 1000 or more plaques. A 10^{-3} dilution should result in a nearly confluent monolayer of infected cells with only a small percentage of the plaques well separated. If virus titers are less than this, then the transfection was not as efficient as it should have been and it may be very difficult to locate a recombinant by visually screening the plates.

3. Score a grid pattern on the bottom of the plate with a sharp instrument to aid in screening the plaques. Scan the plate at a magnification of 30–40X and circle any plaques you suspect may be a recombinant (occlusion-negative, Occ$^-$). Look for plaques that are less refractile and light grey instead of light yellow. After some experience, this can become a very rapid and reliable approach for finding AcMNPV recombinant viruses.

4. Re-examine each circled plaque under an inverted phase microscope at 400X. A green filter on the inverted microscope is used to provide extra contrast. Examine the entire plaque area for the presence or absence of polyhedra. If polyhedra are present, the plaque is not a recombinant. When you first start screening visually for recombinants, use Ac360-β-gal as an Occ$^-$ control (on plates lacking the blue indicator dye, X-Gal). *We can not emphasize enough the importance of this step for individuals unexperienced in discriminating wild-type from recombinant plaques.*

5. When several putative recombinant plaques have been located, check the circled plaques again using a dissecting scope. Place a tiny dot within each circle, directly over the plaque to be picked.

6. Using a 200 μl pipetter and sterile tips, carefully remove the agarose directly over the plaque.

7. If another round of purification is needed, transfer the plaques to tubes containing 1 ml of fresh medium and repeat the plaque assay process. Three rounds of plaque purification are usually sufficient. When the plaques are sufficiently pure, transfer them directly to 25 cm^2 flasks (seeded in advance with 1 x 10^6 cells/flask) and incubate 3–4 days at 27 ° C.

4.4.5 Identifying recombinant viruses by dot hybridization

This procedure is based on that described by Kafatos, et al. [13]. Immunoblot or immunodotblot procedures can be used as well.

1. Seed a flat bottom 96 well microtiter plate with 100 μl of Sf9 cells at a density of 1.5 x 10^5 cells/ml (1.5 x 10^4 cells per well).

2. Pick putative recombinant plaques and place each in a separate well. [7]

3. Incubate at 27 ° C for 2–3 days under humidified conditions.

4. Remove media to a duplicate 96 well plate and store at 4 ° C or freeze at -20 ° C.

5. Lyse the cells in the well by adding 200 μl of 0.5 N NaOH and mixing.

6. Neutralize the solution by adding 20 μl of 10 M NH_4Acetate and mixing.

7. Spot the lysates onto nitrocellulose filters in a dot blot or slot blot apparatus.

   (a) Cut a piece of nitrocellulose and Whatman 3MM paper to size, wet the nitrocellulose in hot water, and equilibrate them both in 1 M NH_4Acetate, 0.02 M NaOH.

   (b) Set up the blotting apparatus as described by its manufacturer. The nitrocellulose should be supported from below by the Whatman filter paper.

   (c) Rinse the wells with 1 M NH_4Acetate 0.02 M NaOH. Make sure all wells filter solutions.

   (d) Apply lysates to the manifold.

   (e) Rinse the wells with 1 M NH_4Acetate 0.02 NaOH.

   (f) Remove the nitrocellulose and wash briefly (2 minutes) in 4X SSC (1X SSC = 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0).

[7] You might also try to enrich for recombinant viruses by doing serial dilutions of virus from a transfection mixture.

8. Air dry the filter and bake for 2 hours at 80 ° C under vacuum.

9. Hybridize with a probe specific for your gene.

10. Identify viral candidates which contain your gene. Purify by several rounds of plaque assays, repeating dot hybridization if necessary. The dilutions for the subsequent round of plaque assays should be from 10^{-2} through 10^{-4}.

4.4.6 Plaque hybridization

This procedure is based on that described by Villareal and Berg[28].

1. Set up plaque assay. Incubate until plaques form, then allow plates to dry overnight or longer by leaving in an unhumidified environment (benchtop or dry incubator).

2. Test a sample plate for proper dryness by lifting the edge of the agarose overlay gently with a sterile spatula; if a milky smear of cells forms at the interface of the agarose and the plate as you lift, the plates are too wet. If plates are too dry agarose will crack and break easily.

3. Prepare the four blotting solutions (A,B,C and D) as listed below:

   Solution A 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl

   Solution B 0.5 N NaOH, 1.5 M NaCl

   Solution C 1.0 M Tris-HCl (pH 7.4), 1.5 M NaCl

   Solution D 0.3 M NaCl, 0.03 M Na Citrate

4. Mark agarose and plate for orientation, using a small cork borer for the agarose and a fine point marker for the plate.

5. Loosen the edges of the agarose with a sterile spatula, and gently lift agarose away from the plate, while holding plate inverted over a larger (100 mm) petri dish. Allow the agarose to roll off the plate and drop (cell side up) onto the petri dish. If necessary, adjust the agarose to return it to its original shape. Store the dishes (containing agarose) in a closed container at 4 ° C, with damp paper towels to provide humidity.

6. Place a dry nitrocellulose (NC) filter (47 mm) on top of the cells remaining in the plate. [8] Add a drop of solution A to secure the filter to the plate.

[8] Note: We have also blotted directly from the agarose instead of the plate. If you prefer this method, you may not want to let the plates dry as the majority of the cells will then stick to the agarose instead of the plate when the overlay is removed. If the plates are dried as described above, the cells will stick primarily to the plate. However, there will be mixing of a small percentage of the cells when the overlay is removed. We have little experience with this alternative, so we do not know if this is a better approach.

Hold the plate up to a light source, and trace the orientation markings from the plate onto the NC filter. Use a blue ball-point pen for best results.

7. Saturate a 47 mm circle of Whatman 3 MM paper with solution A. Place this filter on top of the NC filter in the plate. Tamp the filters down firmly (moving from the center outward) with a large rubber stopper to bring NC filter in contact with cells and force out any air pockets.

8. Wait one minute, then tamp down again and remove Whatman filter. Carefully remove the NC filter and place it (cell side up) on a Whatman filter saturated with solution B. Leave on for 2–3 minutes, and dry on paper towels.

9. Transfer filter to a Whatman filter saturated with solution C. Leave on for 2–3 minutes, and dry on paper towels.

10. Wash NC filter by gently immersing in a petri dish filled with solution D. Remove and dry on paper towels.

11. When all filters have been prepared, bake them for 2 hours at 80 ° C under vacuum.

12. Hybridize filters with a suitable labeled probe.

13. After developing autoradiogram of hybridized filters, circle the positive areas and align to the original agarose layer to determine locations to pick.

14. Pick a fairly large (several mm) area of agarose surrounding each positive, then transfer to 1 ml of medium and plaque purify again.

4.5 Viral DNA isolation

4.5.1 Purification of extracellular virus DNA

Relatively pure viral DNA can be obtained from extracellular virus particles (ECV), separated from infected cell culture medium by centrifugation. *Spodoptera frugiperda* (Sf9) cells infected with AcMNPV (at 2 x10^6 cells/ml) will yield about 1 μg of purified ECV DNA per ml of culture medium after 48 hours p.i. The major problems encountered during purification are degradation of the DNA by mechanical shearing and contaminating nucleases. Also, if the DNA concentration is too high during purification, much of it can be lost during phenol extraction. These difficulties can be avoided with the following procedure adapted from Smith, et al. [19]:

1. Infect *S. frugiperda* cells in flasks or suspension cultures as described in the previous sections. It is usually convenient to purify viral DNA from 100–1000 ml of culture medium.

2. At 36–48 or more hours post-infection, remove the infected cells by pelleting at 2500 rpm for 10 minutes in a low speed centrifuge. Transfer the supernatant to ultracentrifuge tubes and pellet the ECV by spinning for 30 minutes at 100,000 x g (24,000 rpm in an SW-27 rotor).

3. Decant the medium and invert the centrifuge tubes on a paper towel for a few minutes. Wipe the residual medium from the sides of the tubes.

4. Resuspend overnight gently the ECV in 0.1X TE in a small volume. Layer half onto each of two linear 25%-56% sucrose gradients made up in 0.1X TE (typically 11 ml gradients in SW-41 tubes). Centrifuge at 100,000 x g for 90 minutes. Visualize the viral band by illuminating the gradient from the bottom of the tube. Remove the broad viral band using a Pasteur pipet (about 1/3 of the gradient). Dilute with 0.1X TE and pellet at 100,000 x g for 30 minutes (again in an SW-41 tube).

5. Using a 200–1000 μl pipet, resuspend the pellets in a total of 4.5 ml Extraction Buffer. Transfer the suspensions to a 15 ml polypropylene centrifuge tube, then add about 200 μg of proteinase K.

6. Digest at 50 ° C for 1–2 hours. Add 0.5 ml of 10% Sarkosyl and continue to incubate at 50 ° C for an additional 2 hours or, if convenient, overnight.

7. Extract twice with phenol/chloroform/isoamyl alcohol (25:24:1). AcMNPV DNA is relatively sensitive to shearing, so extract by inverting the tubes just fast enough to thoroughly mix the phases, without vigorous shaking. Continue to mix for about 5 minutes, then separate the phases by low speed centrifugation. Carefully transfer the DNA-containing aqueous phase to another tube with a wide mouth 5 or 10 ml pipet.

8. Add 10 ml of absolute ethanol and mix well. The viral DNA should be visible as a cotton-like precipitate. Incubate at -80 ° C for 10 minutes to completely precipitate the DNA. Pellet in a low speed centrifuge (2500 rpm) for 20 minutes.

9. Wash the DNA pellet with cold 90% ethanol. Centrifuge briefly to remove the residual alcohol, removing the last of it with a pipet.

10. Add 500 μl of 0.1X TE, and incubate at 65 ° C for about 15 minutes to help resuspend the DNA. Store the DNA at 4 ° C.

4.5.2 Purification of total DNA from infected cells

Very late in infection ($\geq$48 hours p.i.) approximately 25% of the total nucleic acid content of the infected cell nucleus is viral DNA. This provides a rich source for partially purified viral DNA that is suitable for restriction enzyme and Southern blot hybridization analysis. Cellular RNA and DNA do not greatly interfere with either of these procedures. This is also a very convenient source for DNA when checking newly created recombinant viruses for the presence of a foreign gene by restriction analysis. Approximately 100 μg of total nucleic acids (25 μg viral DNA) can be obtained from 0.5–1.0 x 10^7 infected cells (one 25 cm^2 flask of infected cells). The final nucleic acid preparation will be about 25% viral DNA. Take this into account when doing restriction digests, etc. and adjust volumes accordingly.

1. Seed Sf9 cells at an appropriate density and infect with virus. Late in infection (60–72 hours p.i.), pour off the culture medium and remove any residual medium with a Pasteur pipet.

2. Add 5 ml of Lysis Buffer to each flask, which is in a horizontal position so the buffer contacts the cells. In a few minutes the cells will loosen from the bottom.

   Lysis Buffer

   0.03 M Tris, pH 7.5

   0.01 M Mg Acetate

   1.0% Nonidet P-40

   Add Tris and Mg Acetate to 700 ml distilled water. Adjust pH to 7.5, then add NP-40 and bring volume to 1 liter. Filter to sterilize, then store at 4 ° C.

3. Transfer cell suspension to a 15 ml centrifuge tube and keep on ice for 5 minutes. During this time, vortex 4 or 5 times at full speed for about 15 seconds each time.

4. Pellet the nuclei at about 2000 rpm for 3 minutes in a low speed centrifuge. Discard the supernatant (cytoplasmic fraction).

5. Wash the pelleted nuclei once in cold 1X PBS [3]. Repellet the nuclei.

6. Add 4.5 ml Extraction Buffer to the pellet and mix. Add about 200 μg proteinase K and incubate at 50 ° C for 1 hour.

7. Add 0.5 ml 10% Sarcosyl and continue to incubate at 50 ° C for at least 2 hours.

8. Extract, ethanol precipitate, wash, and resuspend as described earlier for ECV DNA purification.

4.6 Radiolabeling recombinant proteins

A plaque-purified virus can be analyzed for production of proteins under polyhedrin promoter control using the protocol outlined below.

Several controls are necessary to allow valid comparisons of levels of expression of foreign proteins from recombinant baculoviruses. Be sure to include a wild-type (AcMNPV) control and an uninfected cell control. You may also include Ac373-CAT or Ac360-β-gal as controls. It is necessary to disrupt the polyhedra in wild-type controls by adjusting the pH to strong alkaline conditions (0.1 M NaOH final concentration) before adding SDS-PAGE disruption buffer. Without this alkaline disruption, less than 10% of the polyhedrin will be solubilized by boiling in disruption buffer.

1. Seed 6 x 10^5 cells/well in a 24 well plate and let the cells attach for at least one hour.

2. Pipet off the cell-free medium and overlay with medium containing the viral stock. At least 200 μl of medium plus viral stock are necessary to prevent the cells from drying out and dying. Incubate for one hour at 27 ° C.

3. Carefully remove the viral inoculum and replace with 500 μl of complete medium. Alternatively, add 300 μl of complete medium to the 200 μl of inoculum present in the well.

4. Incubate the plate for the desired length of time (usually 48–72 hours for genes under polyhedrin promoter control) at 27 ° C. If your incubator is not humidified, it may be necessary to place your plate in a plastic bag containing a moist paper towel to prevent the medium from evaporating.

5. Remove the cell-free medium from above the cells and transfer to another 24 well plate. If you want to determine whether your recombinant protein is secreted into the medium before this time, transfer the supernatants to microcentrifuge tubes and spin at full speed for 3 minutes to pellet any cellular debris. Transfer about 90% of the supernatant to a new tube and discard the pellet in the old tube. Add an equal volume of 2X SDS-PAGE protein disruption buffer to your supernatant and boil for 3 minutes before freezing for later SDS-PAGE analysis.

6. To the cells, carefully add 200 μl of methionine-free Grace's medium (or Grace's medium lacking any other amino acid). Incubate at 27 ° C for 30–60 minutes so that intracellular pools of methionine are depleted.

7. Remove and discard the starving medium and replace with 200 μl of fresh methionine-free Grace's medium. Discarding the starving medium also serves to get rid of excess fetal bovine serum that complicates SDS-PAGE analysis. To each well, add 10 μCi of ^{35}S-methionine (approximately 1

μl of a 10 mCi/ml stock). Since it is difficult to pipet 1 μl accurately, you can add the correct amount of methionine to Grace's medium before dispensing in 200 μl aliquots, or dilute the methionine 10-fold with Grace's medium and dispense 10 μl to each well separately. In the latter instance, each well will contain 210 μl.

8. Incubate at 27 ° C for the desired length of time (usually 4–6 hours).

9. Harvest the cells for SDS-PAGE analysis. If you do not care to separate intracellular from secreted forms of recombinant protein, add an equal volume of 2X SDS-PAGE protein disruption buffer to each well and mix well with a plastic pipet tip. If you are interested in distinguishing secreted forms of your protein from intracellular proteins, resuspend the contents of the well with a pipet, and transfer to microcentrifuge tubes for separation. The cell pellets can be disrupted with either a 1X protein disruption buffer or with water and an equal volume of 2X protein disruption buffer. Generally, resuspend cell pellets in a volume that corresponds to the original cell density.

10. Boil protein samples for 3 minutes before freezing for later SDS-PAGE analysis.

5 Preparation of insect cell culture media

Two related types of media are recommended for use with Sf9 cells and the Baculovirus expression vector system:

Grace's Antheraea medium [4] (a relatively simple mixture of salts, carbohydrates and amino acids) is available from commercial suppliers [9] (Gibco liquid, catalog number 350-1590 or KC powder, catalog number IM-500) or may be prepared from cell culture quality reagents. Grace's medium is used for short term incubations of cells (rinsing monolayers, seeding cells, transfection, etc.).

TNM-FH [8], a more complete medium suitable for routine growth of cells in monolayer or suspension, is prepared from Grace's medium by the addition of 3.3 g/liter Yeastolate and 3.3 g/liter Lactalbumin Hydrolysate (both available from Difco). All media should be filter sterilized as soon as possible after preparation and should be stored at 4 ° C.

For complete growth medium, add 10% fetal bovine serum (sterile, heat inactivated), and antibiotics [10] (if desired) to each bottle of TNM-FH just before using. We usually *do not* add antibiotics to the medium for maintenance of continuous (uninfected, stock) cultures of Sf9 cells. However, antibiotics are routinely added to the medium for larger volumes of cells grown for experimental purposes.

5.1 Preparation of TNM-FH medium

TNM-FH Medium (for growth of Sf9 cells) may be prepared from 10X stock solutions of the various components. Yeastolate and Lactalbumin Hydrolysate are obtained from Difco Laboratories. The remaining are obtained from Sigma Chemical Co. These stocks may be prepared well in advance and stored as indicated. All components should be added in the order listed, allowing each component to dissolve completely before adding the next one. The purest quality water should be used for all media (preferably double-distilled, pyrogen-free).

- Label all incoming reagents with dates received, then note this date and the lot number on the media preparation sheet each time the reagent is used.
- Number the open bottles in the order they are to be added (1, 2, 3, etc.); this will avoid substituting similar, but not identical, chemicals into the media by mistake.

[9] Gibco Laboratories, 3175 Staley Road, Grand Island, NY 14072. Phone 800-828-6686; NY State 800-462-2555. KC Biologicals, Inc. P.O. Box 14848, Lenexa, KS 22615. Phone 800-255-6032; in Alaska, Hawaii, Puerto Rico, and Kansas call collect 913-888-5020.

[10] For experiments where antibiotics are recommended, we use a combination of gentamycin sulfate (50 μg/ml) and amphotericin B ("Fungizone") (2.5 μg/ml), or a combination of penicillin (50 units/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml).

- Store all media components together, separated from other tissue culture laboratory reagents as much as possible.
- As a routine procedure, prepare large (10 liter) batches of media rather than several small batches.
- *Always* allow cold and dessicated reagents *to equilibrate to room temperature* before opening.
- Check and replace dessicants regularly.
- When handling any reagent, *use aseptic techniques to avoid contamination.* Use a clean sterile spatula for each reagent.

5.1.1 10X Soluble amino acids

Adjust to a final volume of 1 liter with distilled water; store at -20 ° C. If a precipitate forms upon thawing, stir to dissolve all precipitate before using.

	per liter
1. L-Arginine free base	5.5 g
2. L-Aspartic acid	3.5 g
3. L-Asparagine	3.5 g
4. L-Alanine	2.25 g
5. B-Alanine	2.0 g
6. L-Glutamic acid	6.0 g
7. L-Glutamine	6.0 g
8. Glycine	6.5 g
9. L-Histidine	25.0 g
10. L-Isoleucine	0.5 g
11. L-Leucine	0.75 g
12. L-Lysine-HCl	6.25 g
13. L-Methionine	0.5 g
14. L-Proline	3.5 g
15. L-Phenylalanine	1.5 g
16. DL-Serine	11.0 g
17. L-Threonine	1.75 g
18. L-Valine	1.0 g

5.1.2 100X Insoluble amino acids

Dissolve in 10 ml of 2 N HCl, and slowly add 40 ml distilled water. Heat until dissolved. Adjust volume to 100 ml and store at -20 ° C.

	per 100 ml
19. L-Cystine-HCl	0.22 g
20. L-Tryptophan	1.0 g
21. L-Tyrosine	0.5 g

5.1.3 10X Carbohydrates

Dissolve and store at -20 ° C.

	per liter
22. Sucrose	266.8 g
23. Fructose	4.0 g
24. Glucose	7.0 g
25. Malic acid	6.7 g
26. α-Ketoglutaric acid	3.7 g
27. Succinic acid	0.6 g
28. Fumaric acid (disodium salt)	0.637 g

5.1.4 1000X Vitamins

Aliquot in single-use volumes and store at -20 ° C in dark bottles.

	per liter
29. Thiamine-HCl	20.0 mg
30. Riboflavin	20.0 mg
31. D-Ca pantothenate	20.0 mg
32. Pyridoxine-HCl	20.0 mg
33. para-aminobenzoic acid	20.0 mg
34. Folic acid	20.0 mg
35. Niacin	20.0 mg
36. iso-Inositol	20.0 mg
37. Biotin	10.0 mg
38. Choline Chloride	200.0 mg

5.1.5 Preparing 4 liters of 1X medium

- Add 1500 ml of distilled water to a large beaker.
- Add 400 ml of 10X Soluble AA's and stir.
- Add 40 ml of 100X Insoluble AA's and stir.
- Add 400 ml of 10X Carbohydrates and stir.
- Add 4 ml of 1000X Vitamins and stir.
- Add the following salts in the order listed:

	per 4 liters
39. KCl	8.96 g
40. $CaCl_2 \cdot 2H_2O$	5.296 g
41. $MgCl_2 \cdot 6H_2O$	9.12 g
42. $MgSO_4 \cdot 7H_2O$	11.12 g

- In a separate beaker, dissolve the salts below in 400 ml of distilled H_2O (approximately 50 ° C). Cool and add to main solution. (The heating helps to drive off excess CO_2).

	per 4 liters
43. $NaHCO_3$	1.4 g
44. $NaH_2PO_4 \cdot H_2O$	4.052 g

- Bring the volume of the entire mixture up to nearly 4 liters with distilled water and adjust pH to 6.1 using 10 M KOH. Bring volume to 4 liters. Check osmolarity. It should be approximately 315 mOs.

- Up to this point, you have prepared Grace's medium. To convert it into TNM-FH, add the following components, stirring until dissolved:

	per 4 liters
45. TC Yeastolate	13.32 g
46. Lactalbumin hydrolysate	13.32 g

- Filter sterilize the medium with a 0.2 micron filter. Incubate it at 37 ° C for 2–3 days to check sterility. Store at 4 ° C.

- For complete growth medium, add fetal bovine serum to 10% and antibiotics (if desired) before use. Final pH (after adding serum) should be 6.2.

- To prepare special variations such as methionine-free Grace's medium (for radiolabeling), prepare 10X stocks which are missing the undesired component. Adequate labeling of protein with radioactive amino acids can be obtained using Grace's medium; additional components are not needed for relatively short (1–6 hours) radiolabeling experiments.

5.2 10X Stocks: Preparation checklist

Type of stock: _____ Volume Prepared: _____

Date Prepared: _____ Aliquot Size: _____

Prepared By: _____ Number of Bottles: _____

Water Source: _____ Storage Temp: _____

10X Soluble AA's

Date/Lot		Amount
_____	L-Arginine F.B.	_____
_____	L-Aspartic Acid	_____
_____	L-Asparagine	_____
_____	L-Alanine	_____
_____	B-Alanine	_____
_____	L-Glutamic Acid	_____
_____	L-Glutamine	_____
_____	Glycine	_____
_____	L-Histidine	_____
_____	L-Isoleucine	_____
_____	L-Leucine	_____
_____	L-Lysine HCl	_____
_____	L-Methionine	_____
_____	L-Proline	_____
_____	L-Phenylalanine	_____
_____	DL-Serine	_____
_____	L-Threonine	_____
_____	L-Valine	_____

100X Insoluble AA's

Date/Lot		Amount
_____	L-Cystine	_____
_____	L-Tryptophan	_____
_____	L-Tyrosine	_____

10X Carbohydrates & OA's

Date/Lot		Amount
____	Sucrose	____
____	Fructose	____
____	Glucose	____
____	Malic Acid	____
____	a-Ketoglutaric A.	____
____	Succinic Acid	____
____	Fumaric Acid	____

1000X Vitamins

Date/Lot		Amount
____	Thiamine HCl	____
____	Riboflavin	____
____	Ca pantothenate	____
____	Pyridoxine HCl	____
____	p-Aminobenzoic A.	____
____	Folic Acid	____
____	Niacin	____
____	i-Inositol	____
____	Biotin	____
____	Choline Chloride	____

5.3 Media preparation checklist

Batch # _____

Dated _____

Date Prepared _____

Volume Prepared_____

Media source _____

Water source _____

Prepared by _____

Filtered by _____

Date filtered _____

# of bottles _____

Time at 37 C _____

Contaminated_____

Method of filtering_____

Type of filters_____

Amt. NaHCO3 _____

Amt. NaH2PO4 _____

Volume H2O _____

Starting volume H2O _____

Amt. 10X Soluble AA's _____

Amt. 100X Insoluble AA's_____

Amt. 10X Carbohydrates _____

Amt. 1000X Vitamins _____

Amt. KCl _____

Amt. CaCl2 . 2 H2O _____

Amt. MgCl2 . 6 H2O _____

Amt. MgSO4 . 7 H2O _____

Amt. 1X NaHCO3/NaH2PO4 _____

Final volume desired _____

- Volume so far - _____

Amt. H2O needed _____

Vol. 10M KOH to pH 6.1 _____

Osmolarity of Grace's _____

Amt. Yeastolate _____

Amt. Lact. Hydrolysate _____

Osmolarity after
adding serum _____

5.4 Cell and infection testing checklist

CELL TESTING

Pass #	Date	Time	Total Cells	Viable Cells	% viable	Dblg time	Amt. passed	Amt. fresh media
1								
2								
3								
4								
5								
6								

INFECTION TESTING

Date tested _____ Positive after 48 hours _____

5.5 Sources and catalog numbers of media components

Component	Source	Catalog Number
B-Alanine	Sigma	A-7752
L-Alanine	"	A-3534
L-Arginine (free base)	"	A-3784
L-Aspartic Acid	"	A-4534
L-Asparagine (anhydrous)	"	A-4159
L-Cystine	"	C-7777
L-Glutamic Acid	"	G-5638
L-Glutamine	"	G-5763
L-Glycine	"	G-6388
L-Histidine	"	H-9386
L-Isoleucine	"	I-7383
L-Leucine	"	L-1512
L-Lysine (monohydrochloride)	"	L-1262
L-Methionine	"	M-2893
L-Proline	"	P-4655
L-Phenylalanine	"	P-5030
DL-Serine	"	S-5386
L-Threonine	"	T-1645
L-Tryptophan	"	T-0271
L-Tyrosine (free base)	"	T-1020
L-Valine	"	V-6504
a-Ketoglutaric Acid	"	K-1750
Fumaric Acid	"	F-8509
Malic Acid	"	M-1000
Succinic Acid	"	S-9512
d-Biotin	"	B-4639
Ca pantothenate	"	P-2250
Choline Chloride	"	C-7527
Folic Acid	"	F-8758
myo-Inositol	"	I-7508
Niacin	"	N-4126
p-Aminobenzoic Acid	"	A-3659
Pyridoxine HCl	"	P-6280
Riboflavin	"	R-9504
Thiamine	"	T-1270
MgCl2 . 6 H2O	"	M-2393
MgSO4 . 7 H2O	"	M-1880
KCl	"	P-5405
NaH2PO4 (monobasic)	"	S-5011
Fructose	"	F-3510

Glucose	"	G-6138
Sucrose	"	S-9378
NaHCO3	"	S-8875
CaCl2 . 2 H2O	"	C-7902
Yeastolate	Difco	5577-15-5
Lact. Hydrolysate	"	5996-01

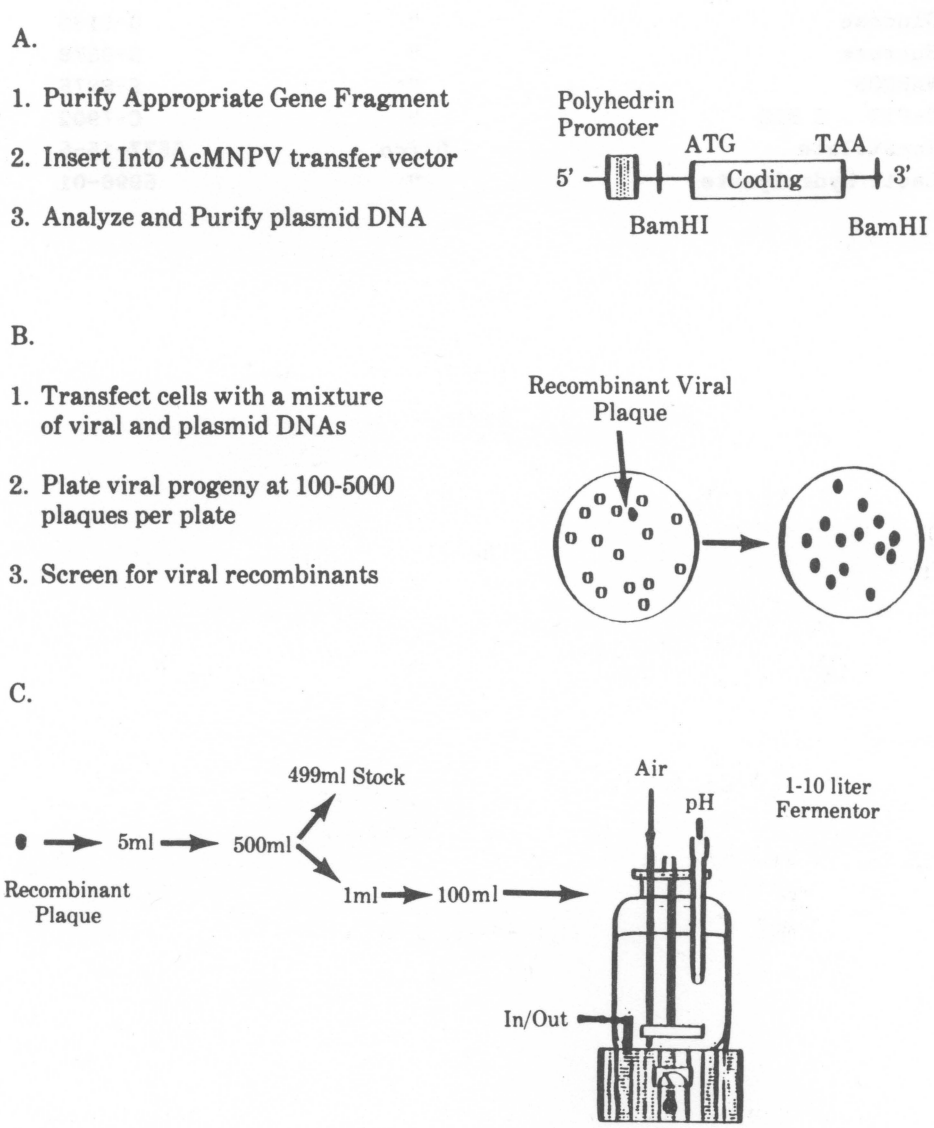

Figure 1: A schematic depicting the procedure and sequence for cloning a foreign gene with its translation start and stop signals and screening for recombinant plaques. The helper-independent recombinant baculovirus produces a lytic infection with the optimal expression and production of a recombinant protein within 48–72 hours post infection. The process of cloning, selection and testing for foreign gene expression should not exceed 3–6 weeks.

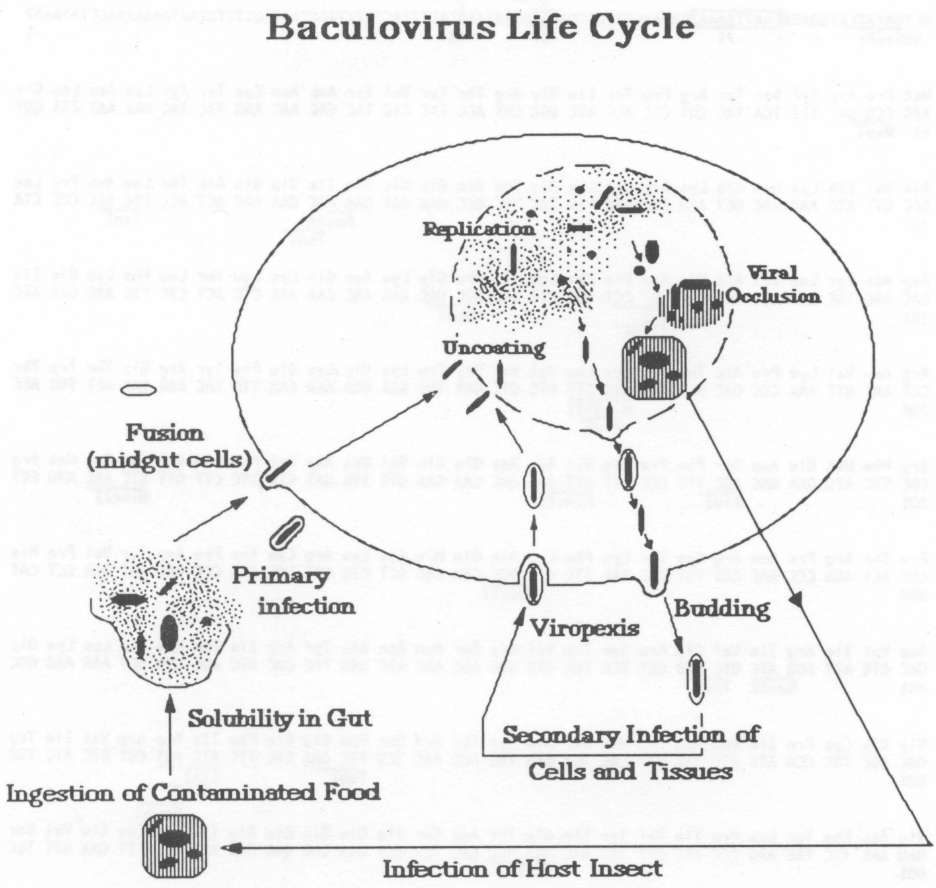

Figure 2: Baculovirus Lytic Cycle. The schematic depicts the unique biphasic life cycle of a typical baculovirus. In the environment a susceptible insect ingests the viral occlusions from a food source. The crystal dissociates in the gut of the susceptible insect to release the infectious virus particles which invade the gut cells, penetrate to the nucleus and uncoat. Viral DNA replication is detected by 6 hours. By 10–12 hours post infection extracellular virus buds from the surface to infect other cells and tissues. Late in infection (18–24 hours post infection) the polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions. The viral occlusions accumulate to large numbers and the cells lyse. The viral occlusions are responsible for horizonal transmission among susceptible insects, the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or the insect host. The polyhedrin gene is not essential for virus infection or replication.

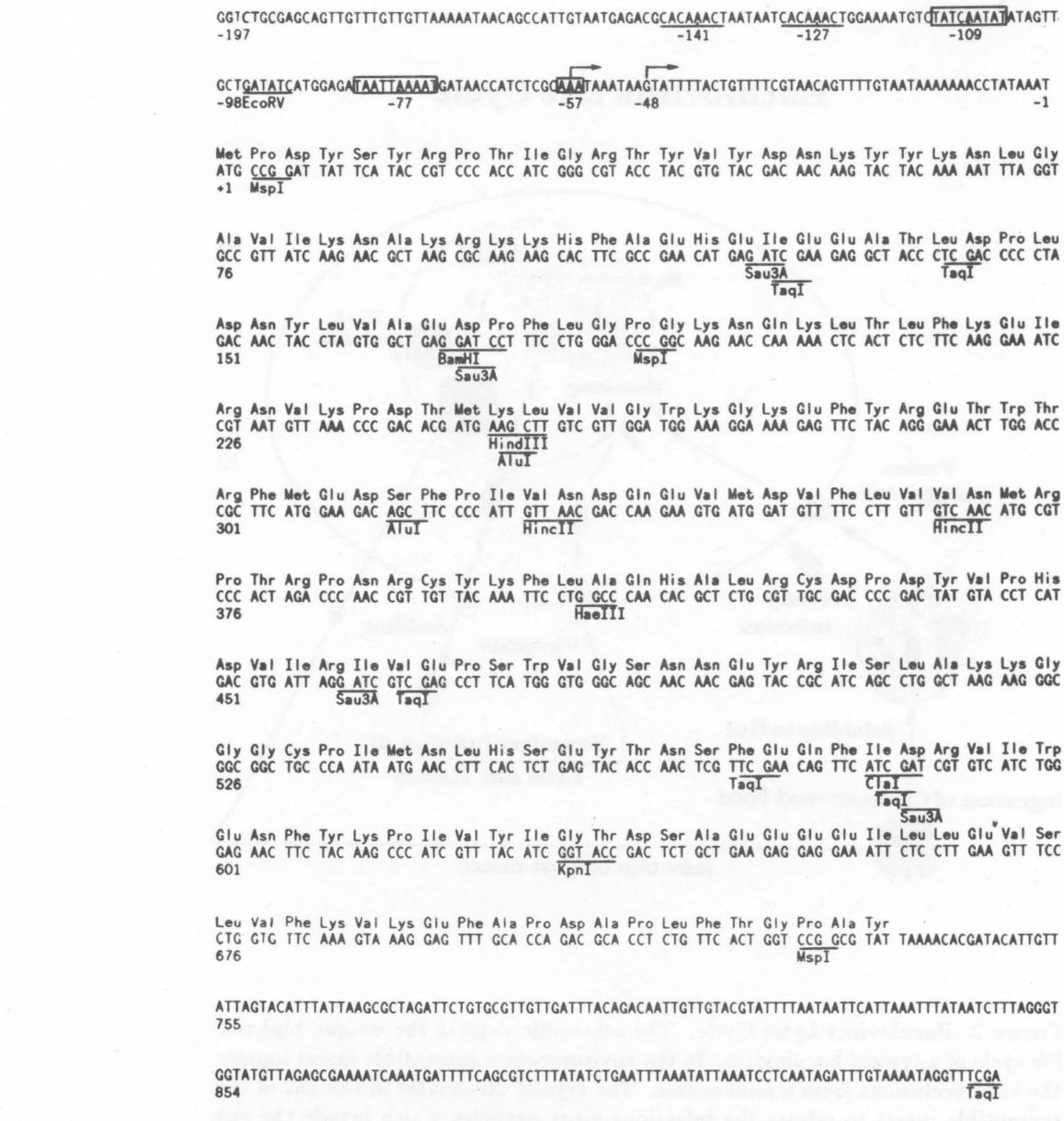

Figure 3: Nucleotide sequence of AcMNPV polyhedrin and flanking 5' and 3' noncoding regions. The + strand is shown. The first nucleotide of the translational start signal ATG is given number +1. The sequence at bp -57 indicates the region containing the 5' end of the mRNA as described by Smith et al. (1983) and the sequence at -48 is the transcription start designated by Posse et al. (1986). The arrow indicates the direction of transcription. The sequences in the open bars at -77 and -109 represent the presumed locations of the canonical TATA box sequences, respectively. The two 8-bp tandem repeats at bp -127 and -141 are underlined. Restriction endonuclease cleavage sites are indicated. This sequence is taken from Iddekinge et al. (1983) and modified to represent the correct sequence at -1 (see Figures 3 and 5).

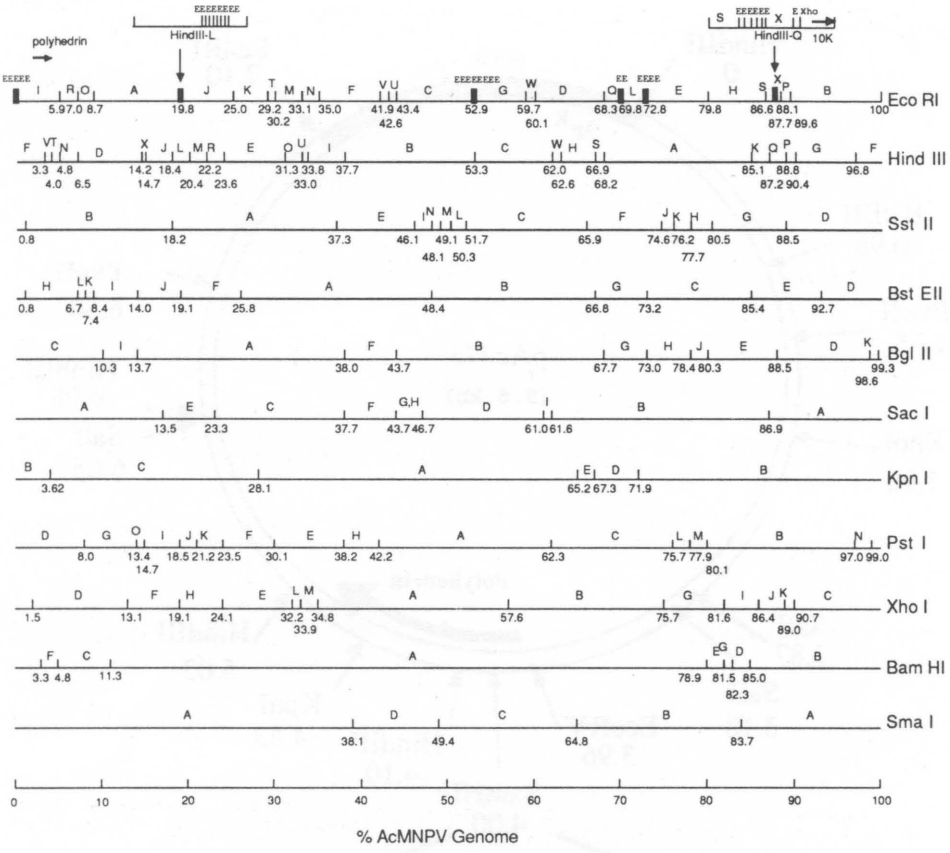

Figure 4: A physical map of the AcMNPV genome with the restriction endonuclease cleavage sites shown.

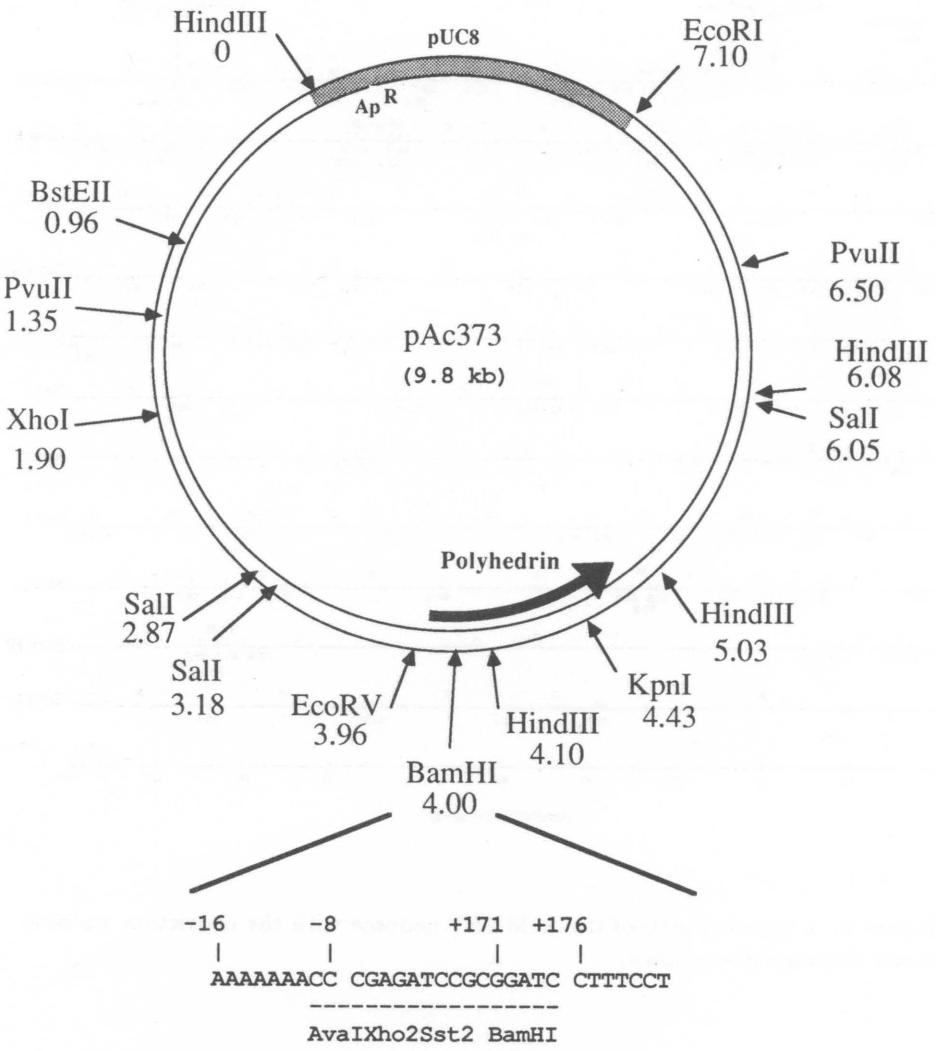

Figure 5: Restriction endonuclease map of the transfer vector pAc373. A unique Bam HI site is present following position -8. There are no cleavage sites for Sma I, Pst I, Bgl II, Xba I or Sst I. Good expression of nonfused foreign proteins requires foreign genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. The polyhedrin polyadenylation signal is present in this plasmid. The nucleotide sequence spanning the deletion from -8 to +171 was determined by V.A. Luckow.

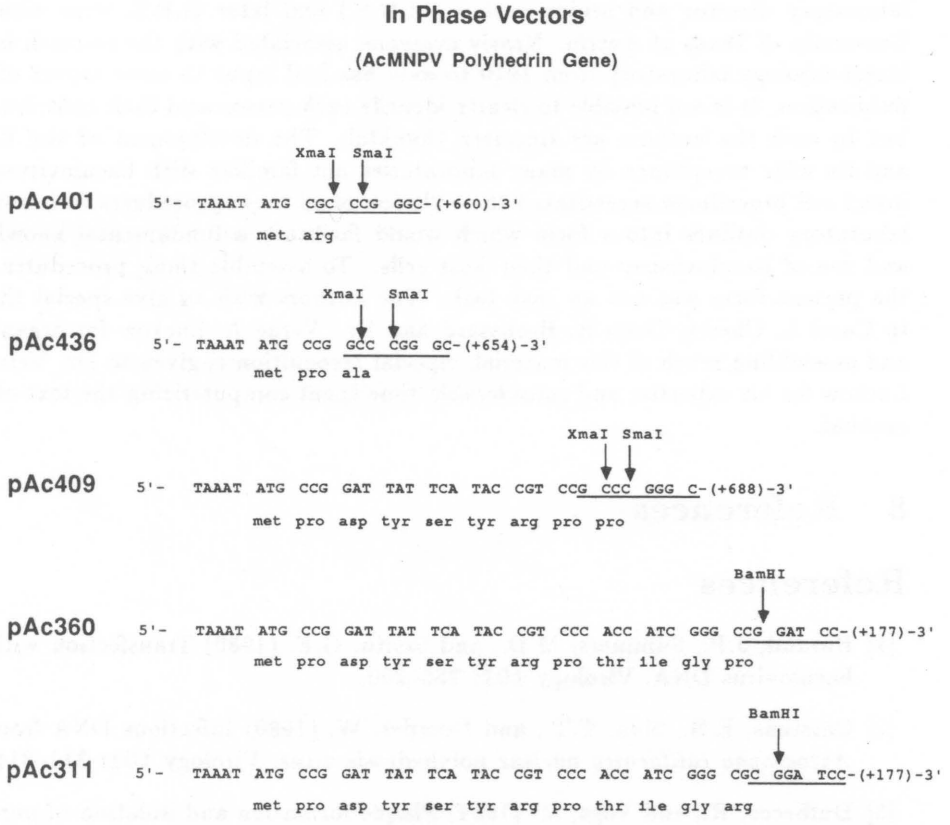

Figure 6: Nucleotide sequence of AcMNPV transfer vectors for the production of polyhedrin/foreign gene fusion proteins. These plasmids have unique Sma I (Xma I) and Bam HI sites downstream from the polyhedrin ATG start codon. These and additional transfer vectors will be described in detail in a forthcoming paper by V.A. Luckow and M.D. Summers. The nucleotide sequences presented above have been determined and/or verified by V.A. Luckow.

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