

Routine Maintenance and Storage of Lepidopteran Insect Cell Lines and Baculoviruses

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Summary

The various methods for maintaining (a.k.a., subculturing, splitting, or passaging) established lepidopteran cell lines are described. Three procedures are presented that are appropriate for different cell lines dependent upon the growth characteristics (in particular, cell attachment properties) of the cells of interest. In addition to the routine maintenance of cells in active culture, methods are also described for both short (low temperature) and long-term (frozen in liquid nitrogen) storage of cell lines, as well as quality control procedures for the cultures. Methods for storing baculoviruses for use in cell cultures and issues of concern when using cell cultures for their production and study are also described.

Key Words: Insect cells; cell line maintenance; cell line storage; liquid nitrogen freezing; cell line testing; sterility; identification; baculovirus storage.

1. Introduction

Given that, as described in Chapter 6, more than 260 distinct cell lines have been reported from more than 50 lepidopteran species, and these cell lines were established by dozens of different researchers, the variability in procedures for handling these cell cultures is almost limitless. Some unique methods have been observed over the years (one postdoc who will remain nameless would throw cultures of a particularly tightly attached cell line across the room to dislodge them for subculturing; the reader may be comforted to hear this individual is no longer a bench scientist), but experience indicates that one of the

three methods described here can be adapted to any insect cell line in existence.

The key to successful maintenance of cell cultures is the use of proper aseptic technique. This is something that is more easily taught in person than by written instructions, but effort is made in the procedures described next to point out some specific actions that should be avoided. A few points that bear repeating: attempts at thrift, such as using a pipet for multiple transfers of materials (medium or cells) can be a bane to cell culture; work with only *one cell strain* in a hood at a time; use a *different container of medium* for each cell strain; and label cultures with the cell strain designation *before* seeding with cells and compare the name on the parent and daughter flasks during the transfer.

The subculture techniques described here were taken nearly verbatim from a paper written 3 yr ago (*1*) because few changes have been made in procedures since that publication. In addition to the procedures outlined next, novices to cell culture should also refer to some general cell culture publications (*2,3*). Although these publications are intended primarily for researchers working with human and other vertebrate cell types, the basic procedures used with insect cell cultures are not dramatically different. In reality, the common use of phosphate buffered medium for lepidopteran cells greatly simplifies their maintenance when compared to mammalian cultures that use carbonate buffers (which necessitate the use of CO₂ incubators) and, as poikiotherms (cold-blooded animals), insects and their cells can survive, and even thrive, at a much broader range of temperatures. Thus, cultures can be maintained under much less stringent temperature regimens or even without an incubator. Additionally, although the author has outlined subculture procedures next for enzymatic detachment of cells (e.g., trypsinization) that is commonly used with vertebrate cells, this procedure is rarely necessary for the generally much less firmly attached cultured insect cells.

In addition to the techniques for handling cell cultures, quality control procedures are described, including methods for assuring cell identity and testing for contaminants. For cell identity, isozyme analysis is used, which is adequate for identifying to the species level. The researcher can avoid the most likely contaminants that are an issue in cell cultures (bacteria and fungi) by use of proper aseptic techniques and *not* using antibiotics in the stock cell cultures. Luckily, the less obvious microbes, such as mycoplasmas are less likely to be a problem with lepidopteran cultures because of the lower incubation temperatures, but the author has provided a method for screening for these agents in any case.

Finally, methods are discussed for storing cell cultures and issues with virus stocks of which the baculovirologist should be aware.

2. Materials

2.1. Supplies for Culturing

1. Mature (late exponential or stationary phase) cell culture.
2. 1-, 2-, 5-, 10-, and 25-mL Sterile sterile pipets.
3. Insect cell culture medium (appropriate for the cell line of interest) (*see Note 1*).
4. Sterile tissue culture flasks (*see Note 2*).
5. Sterile 200- μ L pipet tips.
6. Trypsin diluent: 800 mg NaCl, 20 mg KH_2PO_4 , 20 mg KCl, 150 mg $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 23 mg; Na_2EDTA in demineralized water to 100 mL. Adjust osmotic pressure with 15% NaCl to 350 mOsm/kg and pH to 7.0 with 2 N NaOH. Filter-sterilize through 0.2- μ m filter and store at 4°C.
7. VMF trypsin (virus/mycoplasma free; cell culture tested) (0.05 mg/mL in diluent).
8. Sterile 15-mL centrifuge tubes.
9. 70% Ethanol.
10. Trypan blue solution (0.4%) (optional).

2.2. Quality-Control/Storage Supplies

1. Hoechst 33258 fluorescent stain.
2. Agarose electrophoresis gel (on plastic backing).
3. PHAB Buffer: 50 mM sodium barbital, 10 mM barbital, 10 mM sodium chloride, 1 mM EDTA in demineralized water (*see Note 3*).
4. Malic enzyme substrate solution (ME): 0.27 g DL-Malic malic acid, 2.18 g M Tris (pH 7.5), 25 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 15 mg MTT tetrazolium, 15 mg NADP, 10 mg phenazine methosulfate, 30 mL H_2O (*see Note 3*).
5. Isocitrate dehydrogenase substrate solution (ICD): 2.18 g Tris (pH 7.5), 100 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 50 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 50 mg DL- Na_2 -isocitrate, 15 mg MTT tetrazolium, 15 mg NADP, 10 mg Phenazine methosulfate, 30 mL H_2O (*see Note 3*).
6. Phosphoglucose isomerase substrate solution (PCI): 2.18 g Tris (pH 8.0), 76 mg Na_2EDTA , 120 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 210 mg a-d-glucose-1-phosphate, 6 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL H_2O (*see Note 3*).
7. Phosphoglucomutase substrate solution (PGM): 2.18 g Tris (pH 8.0), 76 mg Na_2EDTA , 120 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mg fructose-6-phosphate, 25 IU glucose-6-phosphate dehydrogenase, 12 mg MTT tetrazolium, 6 mg NADP, 12 mg phenazine methosulfate, 30 mL H_2O (*see Note 3*).
8. Extraction buffer: 10% (v/v) Triton X-100 in 0.6 M Tris-EDTA buffer (pH 7.5) in demineralized water.
9. Stabilization buffer: a proprietary material containing sorbitol and Tris buffer (*see Note 3*).
10. Glycerol.
11. Dimethylsulfoxide (DMSO; sterilized and cell culture tested).

12. Glass freezing ampoules (capped with aluminum foil and heat-sterilized or autoclaved).
13. Long Pasteur pipets, cotton plugged and autoclaved.
14. Aluminum canes for ampoule storage.
15. 1% Methylene red solution in tall graduated cylinder (250-mL size) at 4°C.

2.3. Equipment

1. Biological safety cabinet (preferred) or laminar flow hood.
2. Inverted phase contrast microscope.
3. Mechanical pipetting device (this may be a rubber bulb, a battery powered self-contained unit, or aquarium-pump type).
4. Refrigerated incubator (26–28°C; optional second incubator at 16–18°C).
5. Hemocytometer.
6. Compound light/fluorescent microscope.
7. Electrophoresis power supply (constant 160 V).
8. Electrophoresis temperature-controlled cover and base.
9. Liquid nitrogen Dewar.
10. Ampoule-sealing apparatus or gas/air torch with glass rod.
11. Controlled rate freezing apparatus.

3. Methods

3.1. Stock Cell Cultures: Passage Methods

3.1.1. Preparation of Hood, Examination of Culture, and Labeling of Flask(s)

1. Turn on laminar flow hood and wipe down working surface with approx 2 mL 70% ethanol (keep a 100- to 200-mL squeeze bottle of ethanol next to the hood for this purpose). The hood should be empty at this point. A biological safety cabinet/laminar flow hood is *not* a place to store buffers, pipets or tips, or other supplies. Doing so can disrupt the flow of air through the enclosure, resulting in a contaminated workspace. Additionally, if you use the same hood for work with insect viruses, a good habit would be to use a ultraviolet light for disinfecting the hood overnight and handle the stock cultures of cells early in the day prior to any work with viruses. Additionally, only one cell line should be used in the hood at a time and the working surface should be wiped down with ethanol between cultures. Following this last rule will help avoid cross-contamination or mislabeling of cell lines.
2. Remove a mature cell culture from incubator and examine it with an inverted microscope fitted with a $\times 10$ or 20 phase contrast objective. The medium in the culture should be fairly clear and cells should be somewhat refractive under the microscope (**Fig. 1**). Cells that are hard to see with the microscope owing to a cloudy appearance to the medium suggest a bacterial contamination. Such cultures should be autoclaved and discarded.

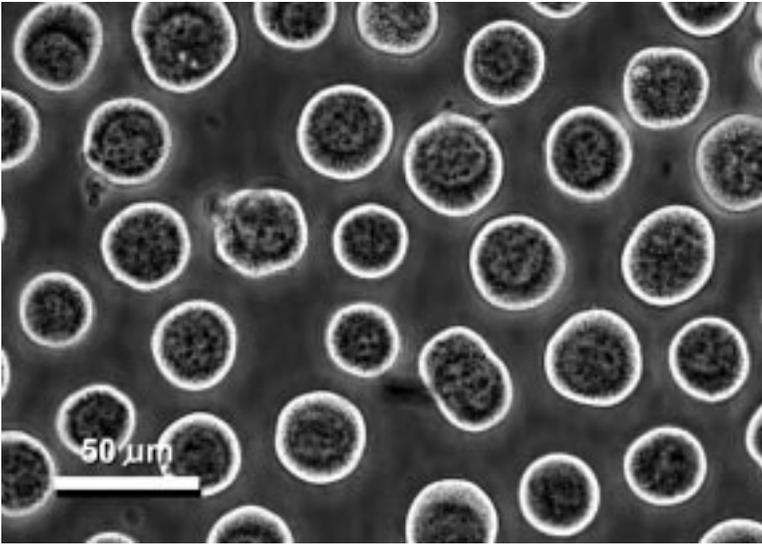


Fig. 1. *Lymantria dispar* fat body cell line, IPLB-LdFB: an example of an insect cell line that grows in suspension. Phase contrast microscopy.

3. Record passage information in a record book. This information should include the date, the name of the culture (cell line designation, passage level, culture ID), the amount to be transferred, and the type, amount and specific source of the culture medium to be used (i.e., the individual bottle of medium should be identified in some way. It is recommended that a bottle of medium be marked with the date when it is first opened and that this date be used as the identifier of the bottle). The amount of the old culture to be added to the new (the split ratio) varies with different cell lines. Some indication of the proper split ratio should be provided by the source of the specific cell line and a range of split ratios should be used during the initial subcultures.
4. Label one or more new 25-cm² tissue culture flask(s) with the date, cell line designation, and passage number. A fine tip permanent marker is useful for this purpose.

3.1.2. Suspension and Loosely Attached Cell Lines

This procedure works well for loosely attached and nonattached cell cultures such as *Trichoplusia ni* Tn-368, IAL-TND1, *Lymantria dispar* IPLB-LdFB (**Fig. 1**), and *Mamestra brassica* IZD-Mb0503.

1. Place the bottle of fresh medium, the mature culture, and the labeled new culture flask(s) in the hood (*see Note 4*). Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5-mL pipet from the box. While holding it

inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything.

2. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. A total volume of 4–5 mL is normally used in a 25-cm² flask. Therefore, if a total volume of 4 mL is being used with a 1:10 split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see Note 5*). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. Although a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.
3. Gently swirl the mature culture to evenly disperse the cells. Stand the culture upright and loosen the cap. Using a new, sterile 1-mL pipet, draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the above example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the flask. Discard the pipet as previously listed.
4. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26–30°C incubator and the medium back in a 4°C refrigerator. Wipe down the working surface of the hood with 70% ethanol.

3.1.3. Attached Cells

This procedure works well for attached cell cultures such as *Spodoptera frugiperda* IPLB-SF21AE, Sf-9, *L. dispar* IPLB-LdEIta, *Anticarsa gemmatalis* UFL-AG286, *Plodia interpunctella* IAL-PID2, *Plutella xylostella* BCIRL-PxHNU3, *T. ni* BTI-TN5B1-4 (sold commercially as High Five[®]) (Tn-5) and IPLB-TN-R² (**Fig. 2**), and *Manduca sexta* MRRL-CH1.

1. Place the mature culture in a refrigerator (4°C) for 20 min.
2. Remove the mature culture and a bottle of fresh medium from the refrigerator and place in the hood with the labeled new culture flask(s) (*see Note 4*).
3. Hold the mature culture in one hand and strike it sharply on the side with the palm of the other hand two or three times to loosen the cells. Set it on end in the hood.
4. Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5-mL pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything.

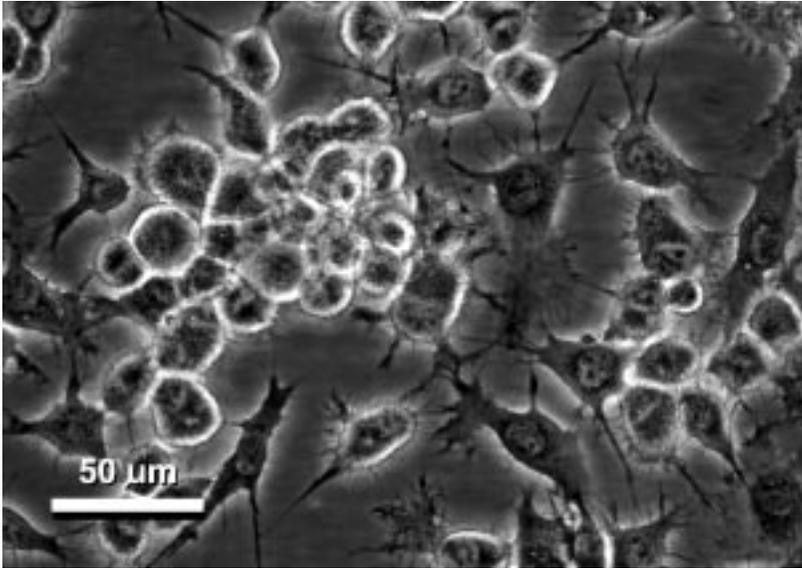


Fig. 2. *Trichoplusia ni* IPLB-TN-R² cells: an attached cell line. Phase contrast microscopy.

5. Remove the cap from the medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. If a total volume of 4 mL is being used in a 25-cm² flask with a 1:10 split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see Note 5*). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. Although a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.
6. Loosen the cap on the mature culture. Using a new, sterile 1-mL pipet, draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the previously listed example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet as previously described.
7. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26–30°C incubator and the medium back in a 4°C refrigerator. Wipe down the working surface of the hood with 70% ethanol.

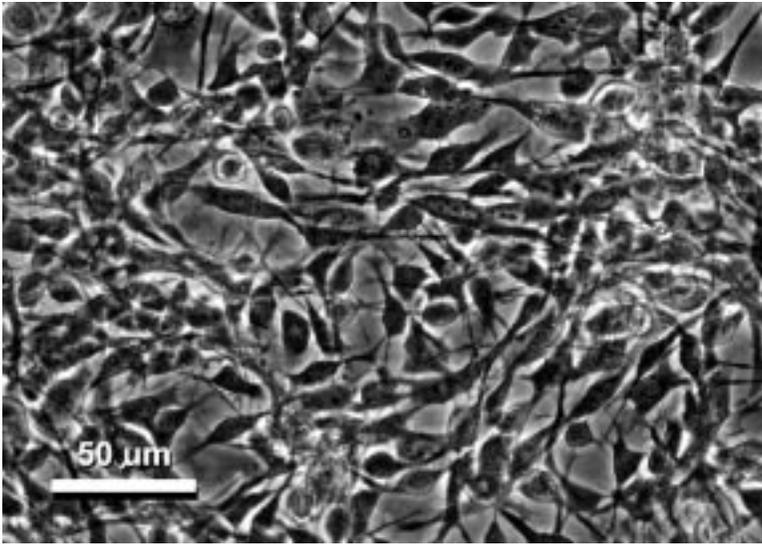


Fig. 3. *Heliiothis virescens* IPLB-HvE1a: firmly attached cells requiring trypsinization. Phase contrast microscopy.

3.1.4. Strongly Attached

This procedure works well for firmly attached cell cultures such as several of the *Heliiothis virescens* lines developed in my laboratory: IPLB-HvT1, IPLB-HvE1A (**Fig. 3**), and IPLB-HvE6A.

3.1.4.1. CELL TRYPsinIZATION

1. Remove the trypsin solution and the diluent from the refrigerator and place them in the hood with the mature culture and a sterile 15-mL test tube.
2. Remove the cap from the test tube and the mature culture. Take a new, sterile 5-mL pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5–10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything. Use the pipet to remove and transfer the medium from the culture to the tube.
3. Loosen the caps on the diluent and trypsin solutions. Remove the cap from the diluent and draw 2 mL of the solution into a new sterile 2-mL pipet. Replace the cap on the diluent and remove the cap from the culture. Slowly release the diluent solution from the pipet letting it wash across the cell monolayer. Draw the diluent solution back into the pipet and transfer it to the tube with the old culture medium (eventually this tube and its contents will be discarded). Some cell lines may benefit from a second rinse with diluent.

4. Take a new 1-mL pipet and transfer 1.0 mL from the trypsin solution to the culture. Replace the cap and tilt the culture flask back and forth to ensure the entire monolayer is wetted by the solution. Set the culture flat on the working surface of the hood and wait 2–5 min. Return the diluent and trypsin solutions to the refrigerator during this waiting period.
5. Tilt the culture once more to ensure the surface is wet, and then remove the cap and pipet out 0.7 mL of the contents into the test tube with the old medium and rinse solution. Replace and tighten the cap on the culture.
6. Wait 5 min more. Tap the flask gently on the hood and hold the culture up to the light to see if the cells are loosened. It is quite apparent when the monolayer has become detached. If it has not, tilt the culture again to wet the cell monolayer, lay it flat and wait 5 min more. Repeat this process until the cells are loose.

3.1.4.2. CELL TRANSFER

3.1.4.2.1. Preferred Procedure

1. Remove a bottle of fresh medium from the refrigerator and place it and the labeled new culture flask(s) in the hood. Loosen the caps on the medium and new culture flask(s).
2. Take a new 5-mL pipet from the box and open as previously described. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. If a total volume of 4 mL is being used in a 25-cm² flask with a 1:10 split, then 3.6 mL fresh medium should be used. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle (*see Note 5*). If more than one new culture is needed, then repeat this procedure for the additional labeled flasks. Although a larger volume pipet can be used to dispense aliquots into several flasks, to avoid contamination, a pipet should never be reused to make additional transfers from the bottle of medium.
3. Loosen the cap on the mature culture. Using a new, sterile 5-mL pipet draw in 5 mL fresh medium and dispense it across the cell surface in the trypsinized culture. Draw the medium back into the pipet and release it a few times to evenly disperse the cell suspension. Although some foaming will occur in this process, care should be taken to minimize this because it can result in more damaged cells. Draw in the appropriate amount of the cell suspension (in the previously listed example, this would be 0.4 mL). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet previously listed.

3.1.4.2.2. *Alternate Procedure.* The previously described procedure assumes that a culture medium containing FBS was used. FBS contains trypsin inhibitors, which stop the activity of the enzyme when the fresh medium is

added. If the cells are being maintained in a serum-free medium, then some serum-containing medium (or some other trypsin-inhibiting solution) should be added at this stage. If it is desired to maintain an essentially serum-free culture, then the medium can be replaced in the new flask(s) with fresh serum-free medium after the cells have had a chance to attach (1–2 h after the culture is initiated).

1. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26–30°C incubator and the medium back in a 4°C refrigerator. Wipe down the working surface of the hood with 70% ethanol.
2. Optional: determination of cell viability (for all cell types). After the cells have been suspended into the medium, whether by mechanical or enzymatic means, 0.2 mL of the cell suspension can be mixed with 0.3 mL PBS and 0.5 mL Trypan blue (final concentration, 0.2% w/v) in a small test tube. Place an aliquot on a hemacytometer and count with the compound microscope. The number of viable cells (those not taking up the stain) can be determined and used for initiating the new culture with a precise number of viable cells. The author found this is a time consuming step that does not greatly improve the probability of maintaining healthy cultures, but this is largely because the author feels confident in recognizing healthy cells just by examining them in the flask with the inverted microscope. Beginners may want to include this step until they gain confidence in their visual inspection of cells.

3.1.5. Backup Cultures

After transferring cells from a mature culture for routine maintenance of a cell line, the left over cells can be left in the original flask and held at a lower temperature as a backup. The author typically uses a 17°C incubator for this purpose (other researchers place them in a laboratory drawer, and so on, i.e., at room temperature). Adding some fresh medium to the mature culture will assure that the cells can be recovered for 2 or 3 wk. Thus, if a daughter culture is discovered to be unhealthy, then the parent or grandparent culture can be used to recover the cells without needing to resort to using cells from long-term storage.

3.2. Quality Control and Testing

The quality of experimental results is directly related to the quality of the cell cultures being used. If the proper technique as previously described is employed and the supplies used are obtained from reputable companies, then there is little reason to think that the results will not be valid. Even so, routine testing of cells can help dispel any suspicions of the experimental results. The regularity of such testing will depend on various factors. In a small lab using a single cell strain, the possible cross-contamination of cells should be nonexist-

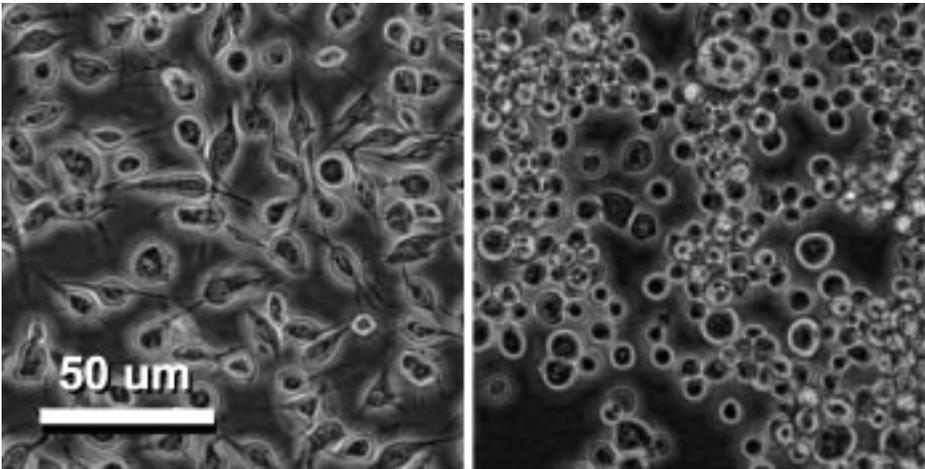


Fig. 4. *Anticarsa gemmatalis* UFL-Ag286 cells. (A) Grown in TC-100 medium, (B) in Ex-Cell 400 serum-free medium.

ent, so cell identity should not be an issue. On the other hand, if several cell lines are kept, then it is essential that a rigorous test be employed. Many cell lines have similar morphologies that can change depending on the medium and other culture conditions that are used (Fig. 4).

3.2.1. Sterility

Cell culture medium provides excellent nutrient properties for the growth of many microorganisms and as long as no antibiotics are added to the medium, it usually becomes apparent quite quickly when a culture or medium is contaminated with bacteria or fungi. Simply incubating an aliquot of the medium is an adequate sterility testing for microbial contamination in most cases. We typically use 100 mL of a 5-L batch of lab-prepared medium and, in my experience, sterility checking of commercially prepared medium has never been necessary. Of course, this requires that you buy your medium from a reputable supplier.

Good cell culture laboratory practice dictates that antibiotics should *not* be used in the cultures used to maintain a cell line. Having an antibiotic in the medium may actually create a greater risk to the cells because a low level microbial contamination could go undetected until antibiotic resistance develops, by which time the problem could have spread to many different cultures being maintained in the laboratory.

Mycoplasmas are a potential problem in cell cultures because of their ability to pass through small pore-sized filters (typically

used for sterilizing solutions such as culture media that contain heat-labile ingredients) and their indistinct appearance under the microscope. Insect cell culturists are fortunate that the most common mycoplasma species that contaminate cell cultures are associated with mammals (especially human or bovine). These organisms thrive at temperatures typically used for mammalian cells (35–37°C), but grow quite poorly at temperatures that are used for insect cells. Steiner and McGarrity (4) purposefully contaminated *Drosophila* cell cultures with mycoplasmas and could no longer detect many of these microbes after normal maintenance for a few weeks. Still, a regular testing schedule will assure the researchers that their cultures are clean. One of the simplest assays involves the use of a fluorescent dye, Hoechst 33258, to look for extra-nuclear DNA:

1. Grow attached cells on glass cover slips.
2. Fix by submersion in 3:1 methanol:acetic acid.
3. Stain with 0.25 µg/mL Hoechst 33258 for 30 min.
4. Mount the cover slips in PBS on a glass slide.
5. Observe with a fluorescent microscope (330–380 nm excitation/440 nm barrier filter).

Evidence of extranuclear fluorescence, especially along the cytoplasmic membrane, is suggestive of mycoplasma contamination.

3.2.2. Identification

Cell line identity is an important issue in cell culture. Nelson-Rees and Daniels (5) brought this point to light in the 1970s when they revealed that many of the normal human diploid cell lines that were being used in research programs around the world were often not normal (typically cells thought to be from normal human tissues were in fact a line of cervical cancer cells known as HeLa) and, in some cases, were not even human cells. Failure to use the proper cell lines can cause unforeseen responses and negate all the experimental results. Techniques such as chromosome banding patterns were commonly used for identifying mammalian cells but, as a rule, Lepidoptera have rather small chromosomes that become fragmented easily, making karyotype analysis nearly impossible. Isozyme profiles were determined to be an effective means of identifying cell cultures to the species level (6). The method described here relates to the use of Innovative Chemistry's AuthentiKit™ system (*see Note 3*).

3.2.2.1. ISOZYMES

1. Use cells in exponential growth (typically a 5- or 6-d old culture of cells maintained on a weekly subculture interval).

2. Suspend into the medium using the appropriate method described in **Subheading 3.1**.
3. Transfer cells to a centrifuge tube and pellet at 1000g for 10 min.
4. Rinse the cells by resuspending in PBS.
5. Centrifuge at 1000g for 10 min.
6. Decant and discard the supernatant.
7. Suspend the pellet into an equal volume of extraction buffer (i.e., a volume equal to the size of the pellet. For a nearly confluent insect cell line in a 25-cm² flask, this is typically between 25 and 100 μ L).
8. Place on ice.
9. After 15 min, disrupt the cells by drawing and expelling the cell suspension into a Pasteur pipet 10 times.
10. Centrifuge at 1000g for 10 min.
11. Mix the supernatant with an equal volume of stabilization buffer. This material can be stored at -20°C for several months. Apply 1 μ L of the cell extract (or a dilution of it, *see Note 6*) to a small depression in an agarose gel.
12. Set the gel into the electrophoresis cover (that has been cooled to 4°C). When fitted onto the base, the two ends of the gel dip into the two buffer chambers containing the positive and negative electrodes, thereby completing the circuit for the electrophoresis.
13. Connect the base to the power supply and electrophoresed for 25 min.
14. At the end of this time, the gel is laid on a flat surface (with the gel side up/plastic backing down) and 0.5 mL of a specific enzyme (ICD, ME, PGI, or PGM) substrate solution is poured onto the gel and spread evenly across the surface by a single pass of a clean glass rod or 5-mL pipet.
15. Place the gel into a prewarmed incubator tray containing moistened filter paper and incubate at $26\text{--}28^{\circ}\text{C}$ for 20 min (*see Note 7*).
16. Rinse the gel in 500 mL demineralized water with at least one change of the water after about 20 min.
17. Place on a warm surface (under 65°C) or simply in the flow of air in a laminar flow hood. (On a warm surface, the gel is typically dry in about 30 min, but if room temperature in the hood is used, it may take more than 1 hr.)
18. Once dry, the gel can be placed on a white sheet of paper and the band positions marked with a fine-tip permanent marker and then the location measured from the position of the extract application. The gel itself can be attached to a lab notebook as a permanent record or scanned for use in publications (**Fig 5**).

3.3. Storage

Keeping a stock of cells in some form of storage is insurance against contamination events, deficient media, or change of cultures over time because of selection of cell types inappropriate to experimental needs (*see Note 8*). As previously mentioned, keeping cells that are left over from subcultures at a lower temperature as a standard protocol is useful for occasional problems, but other options should be considered.

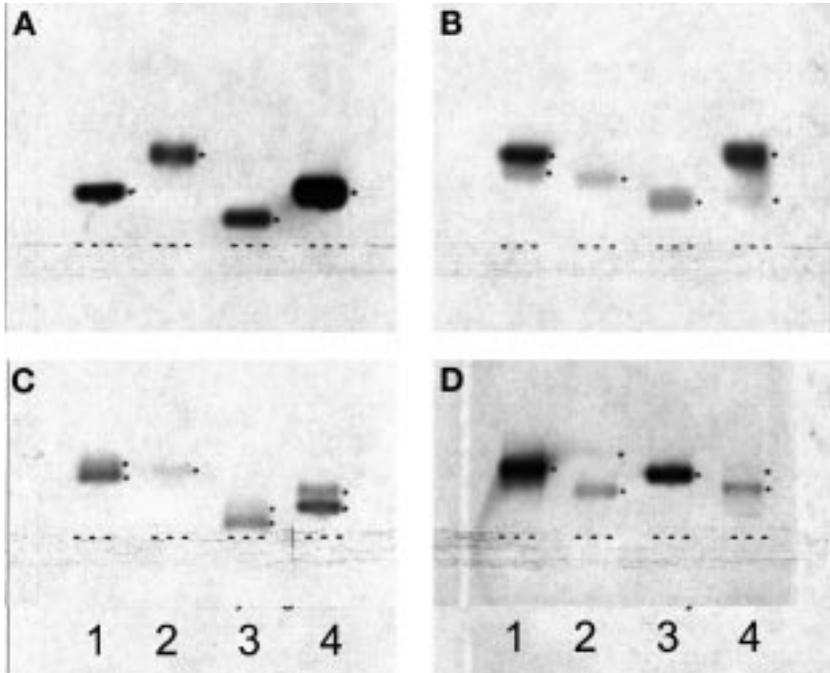


Fig. 5. Isozyme gels. (Lane 1) IPLB-Sf21AE cells; (Lane 2) IPLB-CPB2 cells; (Lane 3) IPLB-DU182E cells; (Lane 4) IPLB-PxE1 cells. (A) Stained for phosphoglucose isomerase (PCI); (B) phosphoglucomutase (PGM); (C) isocitrate dehydrogenase (ICD); and (D) malic enzyme (ME).

3.3.1. Low-Temperature

As mentioned in Chapter 7, lower incubation temperatures (16–18°C) can be a useful means of maintaining different cell types in culture. Winstanley and Crook (7) showed this to be an effective means to maintain susceptibility of some cell lines to specific viruses (in their case, a granulovirus). The same methods described in **Subheading 3.1.** are used for the subculturing. The time interval between splits and the ratios used will need to be determined for each cell line of interest but the author found most insect cell lines can be maintained with a monthly subculture interval and split ratios quite similar to those used when the cells are grown at 26°C.

3.3.2. Liquid Nitrogen

A longer-term solution for storage of cells, especially cultures that are not needed on a routine basis, is liquid nitrogen (LN₂) storage (*see Note 9*). Cells stored in LN₂ are very stable, although the freezing and thawing require special

consideration because cells are damaged during these processes. Taking our cues from other animal cell cultures, we find a freezing rate of about 1°C per min causes the least amount of cell damage, although typically a rapid thawing is the best. In addition to the freezing rate, a cryoprotectant is added to the culture medium. The most common of these are glycerol and DMSO. The author prefers glycerol because it is a common substance naturally used by some cold-tolerant insects, suggesting it would have fewer adverse effects on the cells but the author has also had some cell lines which do not survive freezing with glycerol, so DMSO is a viable alternative. DMSO can have toxic contaminants so a cell-culture tested supply should be used.

3.3.2.1. FREEZING

1. Label the sterilized ampoules with a permanent marker, providing enough information to identify the cells. The author typically uses a brief cell line identity (for example, for the IPLB-Sf21AE cell line, the author would label as SF21) and the passage number.
2. Using the biosafety cabinet/laminar flow hood, suspend a late, exponential phase culture (typically 5 or 6 d old for cultures kept on a weekly subculture interval) by the normal subculture method (**Subheading 3.1.**). The author typically prepares 10 ampoules for freezing from a 75-cm² flask (*see Note 10*).
3. Transfer the cells to a 15-mL sterile centrifuge tube and centrifuge at 50–100g for 5–10 min.
4. While the cells are centrifuging, prepare the freezing medium. This is the normal culture medium (*see Note 11*) plus 10% glycerol (autoclaved) or DMSO (obtain presterilized from most sources of cell culture materials) and should be prepared shortly before use.
5. Decant (and discard) the old medium from the centrifuge tube and resuspend the cell pellet in freezing medium (1 mL per ampoule being prepared—10 mL if using a 75-cm² flask).
6. Use the sterile, cotton-plugged long Pasteur pipets to transfer 1 mL to each glass freezing ampoule (*see Note 12*). Recap with the aluminum foil used during sterilization of the ampoules.
7. Adjust the flame on the sealing apparatus using an empty ampoule of the same type as that used for the cells. Transfer the ampoules to the sealing apparatus a few at a time, removing the aluminum caps shortly before they reach the flame (*see Note 13*).
8. Transfer the ampoules from the sealing apparatus to aluminum canes (the end of the canes should be labeled with a permanent marker with the cell line identity) and place them in the methylene red solution in the graduated cylinder for 30 min.
9. Examine and discard any the ampoules containing the red dye.
10. Place the remaining ampoules on the canes in the freezing apparatus and start the freezing process (*see Note 14*).

11. At the end of the freezing process, transfer the canes to the LN₂ Dewar.
12. Enter the location of the canes into a log book, including full details on the cell line designation, date of freeze, passage number, culture medium, cryoprotectant, number of ampoules prepared, location in the Dewar(s), and person performing the freezing.

3.3.2.2. THAWING

1. Determine the location of the cell line from the log book.
2. Add 10 mL of the appropriate culture medium (the same formulation used for the cells prior to freezing) to a 15-mL sterile centrifuge tube.
3. Prepare a small beaker (preferably plastic, approx 150- to 200-mL size) with water at 30–35°C.
4. While wearing eye protection (a plastic face shield or goggles), use a pair of forceps to remove the ampoule from the aluminum cane and drop it into the warm water bath (*see* the cautions in **Notes 9** and **13**).
5. When thawed, wipe the ampoule with a paper towel dampened with 70% ethanol and, with the ampoule wrapped in the towel, break it at the neck (most currently available ampoules for LN₂ freezing are prescored for easy breaking. If this is not the case, use a triangular file to score the glass before breaking).
6. Use a sterile long Pasteur pipet to transfer the contents of the ampoule to the tube of culture medium.
7. Centrifuge at 50–100g for 5–10 min.
8. Discard the supernatant, resuspend the pellet in 4 mL fresh medium, and transfer to a 25-cm² tissue culture flask and incubate at 26–28°C.
9. Examine the culture at 1- to 3-d intervals and begin regular subculture procedures (**Subheading 3.1.**) once the culture reaches confluency.

3.4. *Baculoviruses*

Hundreds of lepidopteran species have been reported with baculoviruses (**8**), although little is known about the majority of these viruses. Most readers of this book are likely to be using the *Autographa californica* multiple nucleopolyhedrovirus that will be provided as a budded virus (BV) sample as part of an expression vector system. These samples can be applied to a susceptible cell line (*see* Chapter 6) and a typical infection (**Fig. 6**) will be observed within a few days. Much of this book will provide various details about these viruses. The intent with the rest of this chapter is to make the reader aware of some possible issues involved with their use in continuous cell lines.

3.4.1. *Passage Effect*

Two plaque types, named MP (many polyhedra, now called occlusion bodies, OBs) and FP (few polyhedra) (**Fig. 7**) were described in the first description of a baculovirus plaque assay (**9**). Later studies showed that the use of BV progeny over a series of passages in the TN-368 cell line would increase the

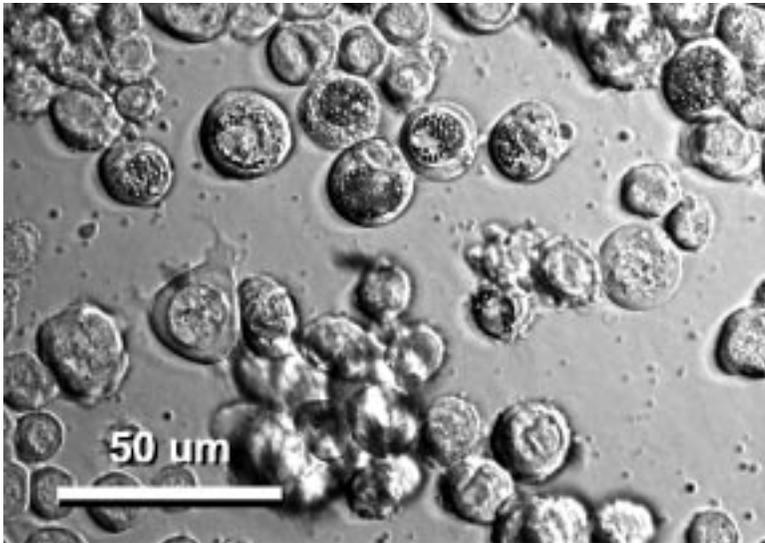


Fig. 6. IPLB-Sf21AE cell line infected with *Autographa californica* multiple nucleopolyhedrovirus 72 h postinfection. Differential interference contrast microscopy.

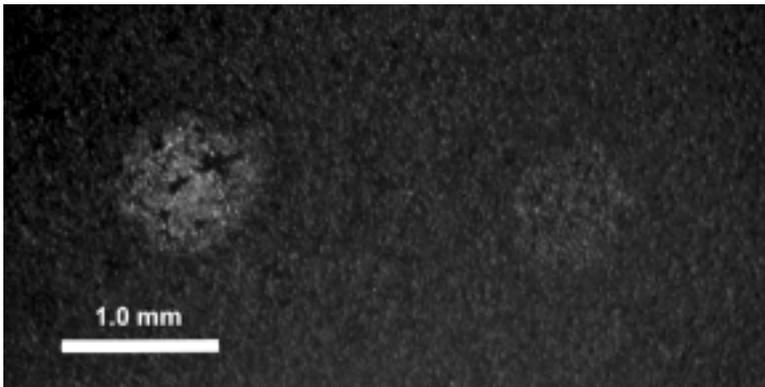


Fig. 7. MP and FP plaques of *Autographa californica* multiple nucleopolyhedrovirus in Tn-368 *cells stained with neutral red.

proportion of FP (10). See Krell (11) for a review of this and other passage effects. The effect can be reversed by feeding OBs to a susceptible insect host (12) or using OB-derived virus (ODV) to infect cell cultures (13). Cell cultures are much less susceptible to ODV, but this may be an effective alternative to

using insects with their potential for having hidden infections with other viruses (**14**).

In addition to the mutant viruses, defective interfering particles can also be created by multiple passages. The use of low multiplicities of infection is an effective method for avoiding these elements (*see* Chapter 1).

3.4.2. Virus Storage

Infectious OBs of baculoviruses are quite stable under a wide range of conditions. Jaques (**15**) could isolate viable virus from soil after more than 4 yr. Lyophilization and storage in airtight containers (even using sealed glass ampoules such as that are previously described for freezing cells) at refrigeration temperatures in the dark is probably the most stable long-term storage for OB. Generally, BV is less stable than OB, but can be stored for years in standard tissue culture medium at 4°C and is even more stable at -85°C (including stability after exposure to multiple freeze/thaw cycles). Jarvis and Garcia (**16**) simply placed cell culture supernatant containing *A. californica* multiple nucleopolyhedrovirus directly into the -85°C freezer without any cryoprotectant addition or any other special procedures. Their medium was supplemented with 10% FBS; it is not known if similar behavior would be observed with serum-free medium. Furthermore, they found that the most detrimental factor in the storage of BV is light. Thus, care should be taken to use lightproof boxes or to wrap the storage containers (either individual tubes or the boxes) with aluminum foil.

4. Notes

1. Grace's insect cell medium was used in the development of the first insect cell lines (**17**) and is still available from a variety of commercial sources. Grace's medium using Hink's additions (**18**) are also commonly available. TC-100 (originally described in the literature as BML-TC/10) (**19**) is a modification of Grace's medium in which a few ingredients subsequently determined to be nonessential or detrimental have been omitted. IPL-41 is a medium developed specifically for IPLB-Sf21AE cells (**20**) and subsequently formed the basis of many of the commercially available serum-free media for lepidopteran cells, e.g., Ex-cell 400 series of media (JRH Biologicals), Sf-900II (Invitrogen/GIBCO), and Insect Express (HyClone). Although many lepidopteran cell lines will grow in more than one of these media, normal practice is to maintain a specific cell line on the same formulation on which it was originally established until experimental evidence is obtained that the cell line properties of interest (e.g., virus susceptibility) is sustained in an alternate formulation.
2. These come in a variety of sizes, typically measured by the area on the growth surface as 12.5-, 25-, 75-cm², and so on, sizes. The author's preference is to use 25-cm² flasks for routine maintenance of cell lines and set up additional cultures

in the larger sizes when cells are needed for experiments. Several manufacturers produce tissue supplies and, in my experience, only personal preferences should be the deciding factor. The author has used BD Falcon®, Corning®, CoStar®, Greiner Cellstar®, and Nunclon® tissue culture labware with no discernable differences in the growth of lepidopteran cell lines. In addition to culture flasks, the same manufacturers produce Petri dishes and multiwell plates. These alternative styles are useful for experiments, but are less desirable for maintenance of cell cultures than culture flasks that can be tightly closed.

3. The gels, buffers, and staining solutions are available as part of Innovative Chemistry's AuthentiKit™. The AuthentiKit is a simple system that uses preformed agarose gels, premeasured buffers, and substrate powders that are dissolved in demineralized water immediately before use. The equipment is also standardized for use in isozyme analysis of cell cultures. Although the reader can substitute lab-prepared versions of all these solutions as described in the methods (most of the formulae shown in this paper are from Tabachnick and Knudson [6] or Brewer [21]), the advantage of the AuthentiKit system is the easy reproducibility in laboratories that do not routinely use native gel electrophoresis for protein separations. If lab-prepared materials are being used, solutions should be prepared fresh each time the isozyme technique is employed. The original AuthentiKit system was designed for mammalian cell cultures and includes seven enzyme substrates. At the same time that this system was being developed, however, Tabachnick and Knudson (6) showed that four enzymes, i.e., ME, ICD, PCI, and PGM, were adequate for identifying a wide variety of insect cell lines to the species level. Innovative Chemistry, Inc. (Marshfield, MA) includes these four enzyme substrates in their catalog, so instead of buying the complete AuthentiKit that includes gels, buffers and substrates for mammalian cell culture identification, one can buy the gels, buffers, and these four substrates as separate items. As an alternative to isozyme analysis, researchers have also used DNA fingerprinting (22) and two-dimensional gel electrophoresis of cellular proteins (23) for identifying insect cell lines. If you routinely use some of these techniques in your laboratory, these are valid alternatives for maintaining confidence in your research materials.
4. Some researchers find it beneficial to allow the culture medium to reach room temperature before using it for transferring cells. The author has not found this to be an issue for the cultures the authors maintains and, in fact, the author feels keeping the medium cold reduces degradation of components, but the reader should be aware of these different opinions. In the case of the procedure for strongly attached cells, because the cells themselves are chilled prior to subculturing, keeping the medium cold is probably advantageous.
5. The author uses a small trashcan lined with an autoclave bag for this purpose. When the bag is full, autoclave it and then seal it in a cardboard box prior to discarding in the trash. Unless you are working with known human or animal pathogens, insect cell cultures are not known to be hazardous. On the other hand, to the general public, tissue culture material can look like medical waste and it is

prudent to put them through a decontamination process (such as autoclaving) for the peace of mind of refuse workers.

6. The instructions with the AuthentiKit suggest checking the level of enzymatic activity in the cell extracts prior to performing the electrophoresis. The procedure essentially involves using a spectrophotometer for determining the activity in the sample. The author generally omits this step because he has learned from experience how much activity can be expected from insect cells for these enzymes. Typically, the author uses a full 1 μL of extract per lane for ME, ICD, and PGM and 1 μL of a 1:2 dilution (using the stabilization buffer for the dilution) for PGI gels.
7. This is another departure from the AuthentiKit instructions. They recommend placing the gels at 37°C. However, their instructions are designed for mammalian cells and this is the normal temperature at which human cells are maintained. The enzyme systems in insects have optimal activity at their typical growth temperatures, 26–28°C.
8. Some reports have indicated that cells lose their ability to replicate baculoviruses (24). This almost certainly is cell line- or virus-dependent and may vary by investigator, being dependent of how carefully the cultures are handled. In my experience, cells become quite stable in their properties by the time they have been maintained in culture for 1 yr. The process of freezing and thawing cells also apply selection pressure to the cultures (selecting for cells that are more resistant to the freezing procedure or the cryoprotectant being used). The main difference between selection owing to freezing and that from long term culture is that the maintenance selection is much more gradual. If cells capable of replicating your virus are lost during the freezing process, then this would become apparent quite quickly so the first time you thawed cells, the event would have already occurred. Unless you are establishing your own cell lines (as described in Chapter 7), the cells you obtain have likely already been through a freeze.
9. Although LN₂ is a useful tool for storing cell cultures, the reader should be careful in its use. At –186°C, exposure to LN₂ immediately freezes flesh, causing severe burns. Additionally, the Dewar should be kept in a well well-ventilated room because nitrogen is continuously being released and can replace the oxygen in an enclosed space. The author strongly encourages the reader to read the material safety data sheet on LN₂ for more details about this material.
10. For most cell lines the author works with, this represents between 1 and 2 million cells and, as long as the recovery from the thawed cells is reasonably good, should be more than the number typically used for initiating a new culture during normal passages. As discussed later, the thawed culture should be examined regularly and subcultured once the cells reach confluency.
11. Cells maintained in serum free media are more susceptible to damage during freezing. Better results may be obtained with these cultures if 5–10% FBS is also added to the freezing medium.

12. Researchers may be tempted to use plastic freezing vials. These can be used if the vials are only stored in the vapor phase of the LN₂ Dewar. This is not practical with the containers I use at my facility.
13. The glass-sealing apparatus the author uses cannot be used effectively in a laminar flow hood. This means the ampoules will be exposed to unfiltered air for a period of time. Fortunately, the opening of these ampoules is fairly small and the heat of the sealing flame will destroy any contaminants that land on the stem, but the author recommends keeping them covered as much as possible until they are sealed. As an alternative to the sealing apparatus, the author has successfully sealed ampoules by hand using a gas-air torch. For this procedure, the author temporarily attaches a glass rod to the tip of the ampoule by melting the glass on both. Then, the ampoule stem is heated about a centimeter lower until the glass melts. With a smooth motion, the rod is twisted and pulled to form the seal. This procedure can be very effective but the author recommends practicing on empty ampoules until you become proficient. *Use of eye protection is very important when working with sealed glass ampoules.* If the interior is heated after the seal is complete, the ampoule can explode. This is also a hazard when removing ampoules from LN₂ because any that are improperly sealed will contain nitrogen that will expand dramatically when warmed to room temperature or above, creating a shower of glass shrapnel.
14. The controlled-rate freezing apparatus lowers the temperature a 1°C per minute. As an alternative, the author has successfully placed the ampoules in a small Styrofoam box and placed it in a -80°C ultralow freezer overnight. The ampoules are then transferred to the LN₂ for storage.

Acknowledgments

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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