Basic Techniques for Mammalian Cell Tissue Culture

Tissue culture technology has found wide application in the field of cell biology. Cell cultures are utilized in cytogenetic, biochemical, and molecular laboratories for diagnostic as well as research studies. In most cases, cells or tissues must be grown in culture for days or weeks to obtain sufficient numbers of cells for analysis. Maintenance of cells in long-term culture requires strict adherence to aseptic technique to avoid contamination and potential loss of valuable cell lines (see *UNIT 1.3*).

An important factor influencing the growth of cells in culture is the choice of tissue culture medium. Many different recipes for tissue culture media are available and each laboratory must determine which medium best suits their needs. Individual laboratories may elect to use commercially prepared medium or prepare their own. Commercially available medium can be obtained as a sterile and ready-to-use liquid, in a concentrated liquid form, or in a powdered form. Besides providing nutrients for growing cells, medium is generally supplemented with antibiotics, fungicides, or both to inhibit contamination. Medium preparation is discussed in *UNIT 1.2*.

As cells reach confluency, they must be subcultured or passaged. Failure to subculture confluent cells results in reduced mitotic index and eventually cell death. The first step in subculturing monolayers is to detach cells from the surface of the primary culture vessel by trypsinization or mechanical means. The resultant cell suspension is then subdivided, or reseeded, into fresh cultures. Secondary cultures are checked for growth, fed periodically, and may be subsequently subcultured to produce tertiary cultures, etc. The time between passaging cells depends on the growth rate and varies with the cell line.

The Basic Protocol describes subculturing of a monolayer culture grown in petri plates or flasks; the Alternate Protocol 1 describes passaging of suspension cultures. Support Protocols describe freezing of monolayer cells, thawing and recovery of cells, counting cells using a hemacytometer, and preparing cells for transport. Alternate Protocol 2 describes freezing of suspension cells.

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

TRYPSINIZING AND SUBCULTURING CELLS FROM A MONOLAYER

BASIC PROTOCOL

A primary culture is grown to confluency in a 60-mm petri plate or 25-cm^2 tissue culture flask containing 5 ml tissue culture medium. Cells are dispersed by trypsin treatment and then reseeded into secondary cultures. The process of removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture.

Materials

Primary cultures of cells
HBSS (*APPENDIX 2A*) *without* Ca^{2+} and Mg^{2+}, 37°C
Trypsin/EDTA solution (see recipe), 37°C
Complete medium with serum: e.g., supplemented DMEM (*APPENDIX 2A*) with 10% to 15% (v/v) FBS (complete DMEM-10 or -15), 37°C

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Sterile Pasteur pipets
37°C warming tray *or* incubator
Tissue culture plasticware or glassware including pipets and 25-cm^2 flasks or 60-mm petri plates, sterile

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Remove all medium from primary culture with a sterile Pasteur pipet. Wash adhering cell monolayer once or twice with a small volume of 37°C HBSS without Ca^{2+} and Mg^{2+} to remove any residual FBS that may inhibit the action of trypsin.

   Use a buffered salt solution that is Ca^{2+} and Mg^{2+} free to wash cells. Ca^{2+} and Mg^{2+} in the salt solution can cause cells to stick together.

   If this is the first medium change, rather than discarding medium that is removed from primary culture, put it into a fresh dish or flask. The medium contains unattached cells that may attach and grow, thereby providing a backup culture.

2. Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer.

3. Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface.

   If cells are not sufficiently detached, return plate to warming tray for an additional minute or two.

4. Add 2 ml 37°C complete medium. Draw cell suspension into a Pasteur pipet and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.

   If cultures are to be split 1/3 or 1/4 rather than 1/2, add sufficient medium such that 1 ml of cell suspension can be transferred into each fresh culture vessel.

5. Add an equal volume of cell suspension to fresh plates or flasks that have been appropriately labeled.

   Alternatively, cells can be counted using a hemacytometer or Coulter counter and diluted to the desired density so a specific number of cells can be added to each culture vessel. A final concentration of ~5×10^4 cells/ml is appropriate for most subcultures.

   For primary cultures and early subcultures, 60-mm petri plates or 25-cm^2 flasks are generally used; larger vessels (e.g., 150-mm plates or 75-cm^2 flasks) may be used for later subcultures.

   Cultures should be labeled with date of subculture and passage number.

6. Add 4 ml fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO_2 incubator.

   If using 75-cm^2 culture flasks, add 9 ml medium per flask.

   Some labs now use incubators with 5% CO_2 and 4% O_2. The low oxygen concentration is thought to simulate the in vivo environment of cells and to enhance cell growth.

   For some media it is necessary to adjust the CO_2 to a higher or lower level to maintain the pH at 7.4.

7. If necessary, feed subconfluent cultures after 3 or 4 days by removing old medium and adding fresh 37°C medium.

8. Passage secondary culture when it becomes confluent by repeating steps 1 to 7, and continue to passage as necessary.

PASSAGING CELLS IN SUSPENSION CULTURE

ALTERNATE PROTOCOL 1

A suspension culture is grown in culture flasks in a humidified 37°C, 5% CO_2 incubator. Passaging of suspension cultures is somewhat less complicated than passaging of monolayer cultures. Because the cells are suspended in medium rather than attached to a surface, it is not necessary to disperse them enzymatically before passaging. However, before passaging, cells must be maintained in culture by feeding every 2 to 3 days until they reach confluency (i.e., until the cells clump together in the suspension and the medium appears turbid when the flask is swirled).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Feed cells as follows every 2 to 3 days until the cultures are confluent:
   a. Remove flask of suspension cells from incubator, taking care not to disturb those that have settled to the flask bottom.
   b. Aseptically remove and discard about one-third of the medium from flask and replace with an equal volume of prewarmed (37°C) medium. If the cells are growing rapidly, add an additional 10% medium by volume in order to maintain optimum concentration of 1×10^6 cells/ml. Gently swirl flask to resuspend cells.
   c. Return flask to incubator. If there is <15 ml of medium in the flask, incubate flask in horizontal position to enhance cell/medium contact.

      At higher volumes of medium the flask can be incubated in the vertical position.

      If using a 25-cm^2 flask, there should be 20 to 30 ml of medium in the flask at confluency.
2. On the days cultures are not being fed, check them by swirling flask to resuspend cells and observing color changes in the medium that indicate good metabolic growth.
3. When cultures are confluent (~2.5×10^6 cells/ml), passage culture as follows:
   a. Remove flask from incubator and swirl flask so that cells are evenly distributed in the medium.
   b. Aseptically remove half of the volume of cell suspension and place into a fresh flask.
   c. Feed each flask with 7 to 10 ml prewarmed medium and return flask to incubator.

      Some labs prefer to split the cells 1:3 or 1:4, although increasing the split ratio will result in a longer interval before subcultures reach confluency.

FREEZING HUMAN CELLS GROWN IN MONOLAYER CULTURES

SUPPORT PROTOCOL 1

It is sometimes desirable to store cell lines for future study. To preserve cells, avoid senescence, reduce the risk of contamination, and minimize effects of genetic drift, cell lines may be frozen for long-term storage. Without the use of a cryoprotective agent freezing would be lethal to the cells in most cases. Generally, a cryoprotective substance such as dimethylsulfoxide (DMSO) is used in conjunction with complete medium for preserving cells at −70°C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage.

Materials

Log-phase monolayer culture of cells in petri plate
Complete medium (e.g., supplemented DMEM, *APPENDIX 2A*)
Freezing medium: complete medium supplemented with 10% to 20% (v/v) FBS and 5% to 10% (v/v) DMSO, 4°C

Benchtop clinical centrifuge (e.g., Fisher Centrific or Clay Adams Dynac) with 45°C fixed-angle or swinging-bucket rotor

1. Trypsinize cells from plate (see Basic Protocol, steps 1 to 4).

   It is best to use cells in log-phase growth for cryopreservation.

2. Transfer cell suspension to a sterile centrifuge tube and add 2 ml complete medium with serum. Centrifuge 5 min at 300 to 350 × *g* (~1500 rpm in Fisher Centrific rotor), room temperature.

   Cells from three or more dishes from the same subculture of the same source can be combined in one tube.

3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet.

4. Add 4 ml of 4°C freezing medium, mix cells thoroughly, and place on wet ice.

5. Count cells using a hemacytometer (see Support Protocol 3). Dilute with more freezing medium as necessary to get a final cell concentration of 10^6 or 10^7 cells/ml.

   To freeze cells from a nearly confluent 25-cm^2 flask, resuspend in ~3 ml freezing medium.

6. Pipet 1-ml aliquots of cell suspension into labeled 2-ml cryovials. Tighten caps on vials.

7. Place vials 1 hr to overnight in a −70°C freezer, then transfer to liquid nitrogen storage freezer.

   Alternatively, freeze cells in a freezing chamber in the neck of a Dewar flask according to manufacturer's instructions. Some laboratories place vials directly into the liquid nitrogen freezer, omitting the gradual temperature drop. Although this is contrary to the general recommendation to gradually reduce the temperature, laboratories that routinely use a direct-freezing technique report no loss of cell viability on recovery.

   Keep accurate records of the identity and location of cells stored in liquid nitrogen freezers. Cells may be stored for many years and proper information is imperative for locating a particular line for future use.

ALTERNATE PROTOCOL 2

FREEZING CELLS GROWN IN SUSPENSION CULTURE

Freezing cells from suspension culture is similar in principle to freezing cells from monolayer. The major difference is that suspension cultures need not be trypsinized.

1. Transfer cell suspension to a centrifuge tube and spin 10 min at 300 to 350 × *g* (~1500 rpm in Fisher Centrific centrifuge), room temperature.

2. Remove supernatant and resuspend pellet in 4°C freezing medium at a density of 10^6 to 10^7 cells/ml.

   Some laboratories freeze lymphoblastoid lines at the higher cell density because they plan to recover them in a larger volume of medium and because there may be a greater loss of cell viability upon recovery as compared to other types of cells (e.g., fibroblasts).

3. Transfer 1-ml aliquots of cell suspension into cryovials and freeze as for monolayer cultures.

THAWING AND RECOVERING HUMAN CELLS

SUPPORT PROTOCOL 2

When cryopreserved cells are needed for study, they should be thawed rapidly and plated at high density to optimize recovery.

CAUTION: Protective clothing, particularly insulated gloves and goggles, should be worn when removing frozen vials or ampules from the liquid nitrogen freezer. The room containing the liquid nitrogen freezer should be well-ventilated. Care should be taken not to spill liquid nitrogen on the skin.

Additional Materials *(also see Basic Protocol)*

70% (v/v) ethanol
Complete medium/20% FBS (e.g., supplemented DMEM-20, *APPENDIX 2A*), 37°C

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Remove vial from liquid nitrogen freezer and immediately place it into a 37°C water bath. Agitate vial continuously until medium is thawed.

   The medium usually thaws in <60 sec.

   Cells should be thawed as quickly as possible to prevent formation of ice crystals that can cause cell lysis. Try to avoid getting water around the cap of the vial.

2. Wipe top of vial with 70% ethanol before opening.

   Some labs prefer to submerge the vial in 70% ethanol and air dry before opening.

3. Transfer thawed cell suspension into a sterile centrifuge tube containing 2 ml warm complete medium/20% FBS. Centrifuge 10 min at 150 to 200 × *g* (~1000 rpm in Fisher Centrific), room temperature. Discard supernatant.

   Cells are washed with fresh medium to remove residual DMSO.

4. Gently resuspend cell pellet in small amount (~1 ml) of complete medium/20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium.

   Cultures are reestablished at a higher cell density than that used for original cultures because there is some cell death associated with freezing. Generally, 1 ml of cell suspension is reseeded in 5 to 20 ml medium.

5. Check cultures after ~24 hr to ensure that cells have attached to the plate.

6. Change medium after 5 to 7 days or when pH indicator (e.g., phenol red) in medium changes color. Keep cultures in medium with 20% FBS until cell line is reestablished.

   If recovery rate is extremely low, only a subpopulation of the original culture may be growing; be especially careful of this when working with cell lines known to be mosaic.

DETERMINING CELL NUMBER AND VIABILITY WITH A HEMACYTOMETER AND TRYPAN BLUE STAINING

SUPPORT PROTOCOL 3

Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments. A hemacytometer is a thick glass slide with a central area designed as a counting chamber.

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from Baxter Scientific (Fig. 1.1.1). The central portion of the slide is the counting platform which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of

a silver footplate on which is etched a 3 × 3–mm grid. This grid is divided into nine secondary squares, each 1 × 1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting.

Accompanying the hemacytometer slide is a thick, even-surfaced coverslip. Ordinary coverslips may have uneven surfaces that can introduce errors in cell counting; therefore, it is imperative that the coverslip provided with the hemacytometer is used in determining cell number.

Cell suspension is applied to a defined area and counted so cell density can be calculated.

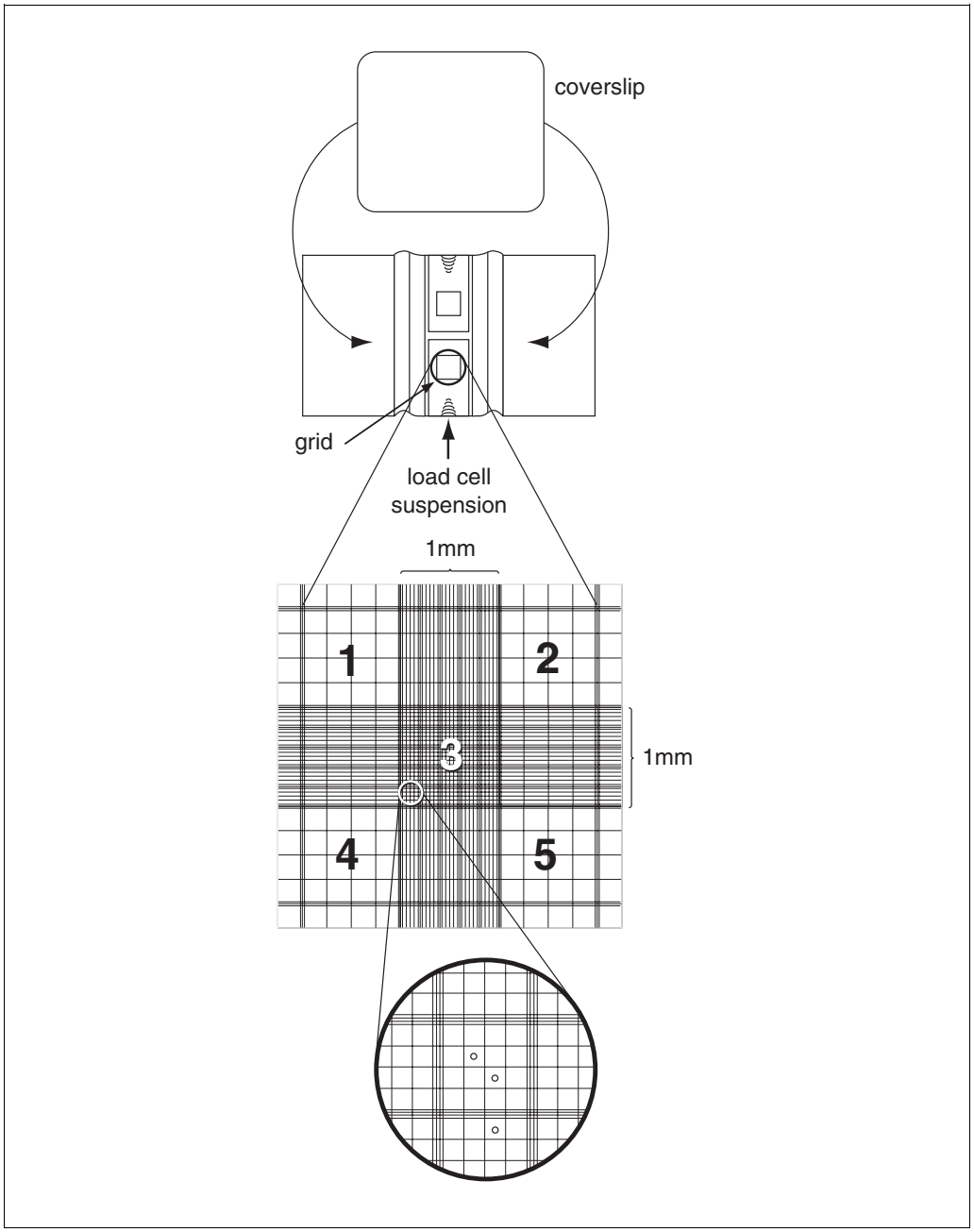

Figure 1.1.1 Hemacytometer slide (Improved Neubauer) and coverslip. Coverslip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipet. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

Materials

70% (v/v) ethanol
Cell suspension
0.4% (w/v) trypan blue *or* 0.4% (w/v) nigrosin prepared in HBSS (*APPENDIX 2A*)

Hemacytometer with coverslip (Improved Neubauer, Baxter Scientific)
Hand-held counter

Prepare hemacytometer

1. Clean surface of hemacytometer slide and coverslip with 70% alcohol.

   Coverslip and slide should be clean, dry, and free from lint, fingerprints, and watermarks.

2. Wet edge of coverslip slightly with tap water and press over grooves on hemacytometer. The coverslip should rest evenly over the silver counting area.

Prepare cell suspension

3. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin (see Basic Protocol, steps 1 to 4).

4. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

   When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1 × 1–mm square is recommended.

Load hemacytometer

5. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.

   Suspension will be drawn under the coverslip by capillary action.

   The hemacytometer should be considered nonsterile. If cell suspension is to be used for cultures, do not reuse the pipet and do not return any excess cell suspension in the pipet to the original suspension.

6. Fill second counting chamber.

Count cells

7. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.

8. View slide on microscope with 100× magnification.

   A 10× ocular with a 10× objective = 100× magnification.

   Position slide to view the large central area of the grid (section 3 in Fig. 1.1.1); this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field. Subdivisions within the large central area are also bordered by three parallel lines and each subdivision is divided into sixteen smaller squares by single lines. Cells within this area should be evenly distributed without clumping. If cells are not evenly distributed, wash and reload hemacytometer.

9. Use a hand-held counter to count cells in each of the four corner and central squares (Fig. 1.1.1, squares numbered 1 to 5). Repeat counts for other counting chamber.

   Five squares (four corner and one center) are counted from each of the two counting chambers for a total of ten squares counted.

   Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculate cell number

10. Determine cells per milliliter by the following calculations:

$$\text{cells/ml} = \text{average count per square} \times \text{dilution factor} \times 10^4$$

$$\text{total cells} = \text{cells/ml} \times \text{total original volume of cell suspension from which sample was taken.}$$

The number 10^4 is the volume correction factor for the hemacytometer: each square is 1 × 1 mm and the depth is 0.1 mm.

Stain cells with trypan blue to determine cell viability

11. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer.

Either 0.4% trypan blue or 0.4% nigrosin can be used to determine the viable cell number. Nonviable cells will take up the dye, while live cells will be impermeable to dye.

12. Count total number of cells and total number of viable (unstained) cells. Calculate percent viable cells as follows:

$$\%\ \text{viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

13. Decontaminate coverslip and hemacytometer by rinsing with 70% ethanol and then deionized water. Air dry and store for future use.

SUPPORT PROTOCOL 4

PREPARING CELLS FOR TRANSPORT

Both monolayer and suspension cultures can easily be shipped in 25-cm^2 tissue culture flasks. Cells are grown to near confluency in a monolayer or to desired density in suspension. Medium is removed from monolayer cultures and the flask is filled with fresh medium. Fresh medium is added to suspension cultures to fill the flask. *It is essential that the flasks be completely filled with medium to protect cells from drying if flasks are inverted during transport.* The cap is tightened and taped securely in place. The flask is sealed in a leak-proof plastic bag or other leak-proof container designed to prevent spillage in the event that the flask should become damaged. The primary container is then placed in a secondary insulated container to protect it from extreme temperatures during transport. A biohazard label is affixed to the outside of the package. Generally, cultures are transported by same-day or overnight courier.

Cells can also be shipped frozen. The vial containing frozen cells is removed from the liquid nitrogen freezer and placed immediately on dry ice in an insulated container to prevent thawing during transport.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Trypsin/EDTA solution

Prepare in sterile HBSS or 0.9% (w/v) NaCl:
0.25% (w/v) trypsin
0.2% (w/v) EDTA
Store ≤1 year (until needed) at −20°C

Specific applications may require different concentrations of trypsin; the appropriate protocols should be consulted for details.

Trypsin/EDTA solution is available in various concentrations including 10×, 1×, and 0.25% (w/v). It is received frozen from the manufacturer and can be thawed and aseptically divided into smaller volumes. Preparing trypsin/EDTA from powdered stocks may reduce its cost; however, most laboratories prefer commercially prepared solutions for convenience.

EDTA (disodium ethylenediamine tetraacetic acid) is added as a chelating agent to bind Ca^{2+} *and* Mg^{2+} *ions that can interfere with the action of trypsin.*

COMMENTARY

Background Information

At its inception in the early twentieth century, tissue culture was applied to the study of tissue fragments in culture. New growth in culture was limited to cells that migrated out from the initial tissue fragment. Tissue culture techniques evolved rapidly, and since the 1950s culture methods have allowed the growth and study of dispersed cells in culture (Freshney, 1993). Cells dispersed from the original tissue can be grown and passaged repeatedly to give rise to a relatively stable cell line.

Four distinct growth stages have been described for primary cells maintained in culture. First, cells adapt to the in vitro environment. Second, cells undergo an exponential growth phase lasting through ~30 passages. Third, the growth rate of cells slows, leading to a progressively longer generation time. Finally, after 40 or 50 passages, cells begin to senesce and die (Lee, 1991). It may be desirable to study a particular cell line over several months or years, so cultures can be preserved to retain the integrity of the cell line. Aliquots of early-passage cell suspensions are frozen, then thawed and cultures reestablished as needed. Freezing cultures prevents changes due to genetic drift and avoids loss of cultures due to senescence or accidental contamination (Freshney, 1993).

Cell lines are commercially available from a number of sources, including the American Type Culture Collection (ATCC) and the Human Genetic Mutant Cell Repository at the Coriell Cell Repository (CCR; *SUPPLIERS APPENDIX*). These cell repositories are a valuable resource for researchers who do not have access to suitable patient populations.

Critical Parameters

Use of aseptic technique is essential for successful tissue culture. Cell cultures can be contaminated at any time during handling, so precautions must be taken to minimize the chance of contamination. All supplies and reagents that come into contact with cultures must be sterile and all work surfaces should be kept clean and free from clutter.

Cultures should be 75% to 100% confluent when selected for subculture. Growth in culture will be adversely affected if cells are allowed to become overgrown. Passaging cells too early will result in a longer lag time before subcultures are established. Following dissociation of the monolayer by trypsinization, serum or medium containing serum should be added to the cell suspension to stop further action by trypsin that might be harmful to cells.

When subculturing cells, add a sufficient number of cells to give a final concentration of ~5×10^4 cells/ml in each new culture. Cells plated at too low a density may be inhibited or delayed in entry into growth stage. Cells plated at too high a density may reach confluence before the next scheduled subculturing; this could lead to cell loss and/or cessation of proliferation. The growth characteristics for different cell lines vary. A lower cell concentration (10^4 cells/ml) may be used to initiate subcultures of rapidly growing cells, and a higher cell concentration (10^5 cells/ml) may be used to

initiate subcultures of more slowly growing cells. Adjusting the initial cell concentration permits establishment of a regular, convenient schedule for subculturing—e.g., once or twice a week (Freshney, 1993).

Cells in culture will undergo changes in growth, morphology, and genetic characteristics over time. Such changes can adversely affect reproducibility of laboratory results. Nontransformed cells will undergo senescence and eventual death if passaged indefinitely. The time of senescence will vary with cell line, but generally at between 40 and 50 population doublings fibroblast cell lines begin to senesce. Cryopreservation of cell lines will protect against these adverse changes and will provide a backup in case of contamination.

Cultures selected for cryopreservation should be in log-phase growth and free from contamination. Cells should be frozen at a concentration of 10^6 to 10^7 cells/ml. Cells should be frozen gradually and thawed rapidly to prevent formation of ice crystals that may cause cells to lyse. Cell lines can be thawed and recovered after long-term storage in liquid nitrogen. The top of the freezing vial should be cleaned with 70% alcohol before opening to prevent introduction of contaminants. To aid in recovery of cultures, thawed cells should be reseeded at a higher concentration than that used for initiating primary cultures. Careful records regarding identity and characteristics of frozen cells as well as their location in the freezer should be maintained to allow for easy retrieval.

For accurate cell counting, the hemacytometer slide should be clean, dry, and free from lint, scratches, fingerprints, and watermarks. The coverslip supplied with the hemacytometer should always be used because it has an even surface and is specially designed for use with the counting chamber. Use of an ordinary coverslip may introduce errors in cell counting. If the cell suspension is too dense or the cells are clumped, inaccurate counts will be obtained. If the cell suspension is not evenly distributed over the counting chamber, the hemacytometer should be washed and reloaded.

Anticipated Results

Confluent cell lines can be successfully subcultured in the vast majority of cases. The yield of cells derived from a monolayer culture is directly dependent on the available surface area of the culture vessel (Freshney, 1993). Overly confluent cultures or senescent cells may be difficult to trypsinize, but increasing the time of trypsin exposure will help dissociate resistant cells. Cell lines can be propagated to get sufficient cell populations for cytogenetic, biochemical, and molecular analyses.

It is well accepted that anyone can successfully freeze cultured cells; it is thawing and recovering the cultures that presents the problem. Cultures that are healthy and free from contamination can be frozen and stored indefinitely. Cells stored in liquid nitrogen can be successfully thawed and recovered in >95% of cases. Several aliquots of each cell line should be stored to increase the chance of recovery. Cells should be frozen gradually, with a temperature drop of ~1°C per minute, but thawed rapidly. Gradual freezing and rapid thawing prevents formation of ice crystals that might cause cell lysis.

Accurate cell counts can be obtained using the hemacytometer if cells are evenly dispersed in suspension and free from clumps. Determining the proportion of viable cells in a population will aid in standardization of experimental conditions.

Time Considerations

Establishment and maintenance of mammalian cell cultures require a regular routine for preparation of media and feeding and passaging of cells. Cultures should be inspected regularly for signs of contamination and to determine if the culture needs feeding or passaging.

Literature Cited

Freshney, R.I. 1993. Culture of Animal Cells. A Manual of Basic Techniques, 3rd ed. Wiley-Liss, New York.

Lee, E.C. 1991. Cytogenetic Analysis of Continuous Cell Lines. *In* The ACT Cytogenetic Laboratory Manual, 2nd ed. (M.J. Barch, ed.) pp. 107-148. Raven Press, New York.

Key Reference

Lee, 1991. See above.

Contains pertinent information on cell culture requirements including medium preparation and sterility. Also discusses trypsinization, freezing and thawing, and cell counting.

Contributed by Mary C. Phelan
Greenwood Genetic Center
Greenwood, South Carolina