Mammalian Cell Tissue Culture Techniques

Katy Phelan[1] and Kristin M. May[2]

[1]Florida Cancer Specialists and Research Institute, Fort Myers, Florida
[2]Genetic Diagnostic Laboratory, Children's Hospital at Erlanger, Chattanooga, Tennessee

Cultured mammalian cells are used extensively in molecular biology studies. A number of special skills are required in order to preserve the structure, function, behavior, and biology of cells in culture. This appendix describes the basic skills required to maintain and preserve cell cultures: maintaining aseptic technique, preparing media with the appropriate characteristics, passaging, freezing and storage, recovering frozen stocks, and counting viable cells. © 2017 by John Wiley & Sons, Inc.

Keywords: aseptic technique • freezing cells • medium • passaging cells • tissue culture

How to cite this article:
Phelan, K. and May, K.M. 2017. Mammalian cell tissue culture techniques. *Curr. Protoc. Mol. Biol.* 117:A.3F.1-A.3F.23.
doi: 10.1002/cpmb.31

INTRODUCTION

Tissue culture technology has found wide application in the field of molecular biology. Cell cultures are utilized in biochemical, cytogenetic, and molecular laboratories for research and diagnostic purposes. 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 (Coté, 1998).

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 its 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.

As monolayer cultures reach confluency, they must be subcultured or passaged. Failure to subculture confluent cells results in reduced mitotic index and eventually in cell death. The first step in subculturing 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 and fed periodically, and may be subsequently subcultured to produce tertiary cultures, etc. The time between passaging of cells varies with the cell line and depends on the growth rate.

In contrast to monolayer cultures which senesce over time, immortalized cells have an infinite life span. Cells can be immortalized by infection with a transforming virus

Current Protocols in Molecular Biology A.3F.1-A.3F.23, January 2017
Published online January 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmb.31

containing an oncogene such as mutant *ras* (Freshney, 2010). Transformed cells provide a continuous supply of cells from the same source. While use of immortal cell lines will be discussed, the methods of transforming cells into immortalized cell lines is beyond the scope of this appendix.

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

CAUTION: When working with human blood cells, or infective 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.

ASEPTIC TECHNIQUE AND BIOSAFETY PRACTICES

It is essential that aseptic technique be maintained when working with cell cultures. Aseptic technique involves a number of precautions to protect both the cultured cells and the laboratory worker from infection.

The laboratory worker must realize that all biological materials handled in the lab are potentially infectious and should be handled with caution. Protective apparel such as gloves, lab coats or aprons, and eyewear should be worn when appropriate. The investigator must be familiar with all institutional and governmental policies and regulations regarding biosafety practices. In the United States, the Centers for Disease Control and Prevention publish criteria for various biosafety levels in laboratory work and standards for work practices at each level (U.S. Department of Health and Human Service, 2009). Care should be taken when handling sharp objects such as needles, scissors, scalpel blades, and glass that could puncture the skin. Disposable scalpels with retractable blades are recommended to reduce the risk of infection and injury following sample handling and during clean-up procedures. Sterile disposable plastic supplies may be used to avoid the risk of broken or splintered glass.

Contamination can occur at any step in handling cultured cells. Care should be taken to maintain the sterility of petri plates, pipets, and flasks that are used for tissue culture. The use of disposable, sterile culture supplies is convenient and has virtually eliminated the need to flame-sterilize instruments and vessels used for tissue culture. The initial cost of purchasing these single-use supplies outweighs the expense associated with the time and effort of washing, packaging, and sterilizing nondisposables. Good sterile technique must be followed. For example, if a sterile pipet tip should come into contact with the bench top or other nonsterile surface, the pipet tip should be discarded and a fresh one obtained. If disposables are not an option, the necks of sterile containers, such as bottles and flasks, and the tips of pipets should be passed through a flame before the pipet is introduced into the container. After pipetting, the neck of the bottle or flask should again be flamed. If work is performed in a laminar flow biosafety cabinet, there is no need for an open flame. The CDC and most, if not all, manufacturers of biosafety cabinets, are opposed to the practice. Use of a gas or alcohol burner in a biosafety cabinet disrupts the flow of the air, which may jeopardize the sterility of the work environment. In addition, the resulting heat buildup may create problems in the working area and damage the HEPA air filter within the cabinet (U.S. Department of Health and Human Services, 2009).

Certain instruments used for tissue culture (forceps, scissors, scalpels, and in some instances, glass bottles) may require autoclave sterilization prior to initial use (Coté, 1999a). The autoclave relies on pressurized steam to destroy microorganisms. Instruments to be autoclaved should be thoroughly washed and dried, then packaged if necessary to ensure that sterility will be maintained after removal from the autoclave until use. Indicator tape or autoclave bags with indicator strips should be used to document that the items have been autoclaved. The indicator tape demonstrates that the item has been autoclaved, but does not ensure sterility.

Historically, if instruments such as forceps, tweezers, scissors, or scalpels were going to be re-used to handle several sequential specimens, they were rapidly sterilized between uses by dipping in 70% alcohol and flaming. Other methods of rapid decontamination are now available. For example, instruments can be inserted into a bench top sterilizing unit that contains a bed of glass beads at a constant high temperature. The beads can be cleaned and re-used. Dry heat decontamination occurs through heat transfer from the glass beads to the instruments. The inserted parts of small instruments are dry sterilized within ~15 sec, while larger instruments may take as long as 1 min. Due to the lack of a method for routine monitoring of the degree of sterilization by this method, this type of sterilizer should be used for research purposes only.

Although tissue culture work can be done on an open bench if aseptic methods are strictly enforced, many labs prefer to perform tissue culture work in a room or low-traffic area reserved specifically for that purpose. As stated earlier, it is imperative that the researcher be familiar with all applicable institutional and government regulations regarding work with the relevant cell type and its infectious potential. At the very least, biological safety cabinets are recommended to protect the cultures as well as the laboratory worker. In a laminar flow hood, the flow of air protects the work area from dust and contamination and acts as a barrier between the work surface and the worker. Many different styles of safety hoods are available, and the laboratory should consider the types of samples being processed and the types of potential pathogenic exposure in making a selection. Manufacturer recommendations should be followed regarding routine maintenance checks on air flow and filters. For day-to-day use, the cabinet should be turned on for at least 5 min prior to beginning work. All work surfaces both inside and outside of the hood should be kept clean and disinfected daily and after each use. A 10% household bleach solution, 70% alcohol, an iodophor, a quaternary ammonium compound, or commercially available liquid disinfectants can be used.

Some safety cabinets are equipped with ultraviolet (UV) lights for decontamination of work surfaces. However, the Centers for Disease Control (CDC), the National Institutes of Health (NIH), and the American Biological Safety Association agree that UV lamps are not required and are not recommended for use in biological safety cabinets (Burgener, 2006). This is based on their questionable effectiveness in decontamination and the hazards to laboratory personnel from accidental exposure. UV lamps may produce a false sense of security, as they maintain a visible blue glow long after their germicidal effectiveness is lost. Effectiveness diminishes over time as the glass tube gradually loses its ability to transmit short UV wavelengths, and may also be reduced by dust on the glass tube, distance from the work surface, temperature, and air movement. Even when the UV output is adequate, the rays must directly strike a microorganism in order to kill it; bacteria or mold spores hidden below the surface of a material or outside the direct path of the rays will not be destroyed. Another rule of thumb is that anything that can be seen cannot be killed by UV. UV lamps will only destroy microorganisms such as bacteria, viruses, and mold spores; they will not destroy insects or other large organisms. Aside from their general ineffectiveness, UV lights pose a safety hazard, as exposure can cause damage to the eyes and skin (Burgener, 2006). Other researchers feel that UV light,

Table A.3F.1 Working Concentrations of Antibiotics and Fungicides for Mammalian Cell Culture

Additive	Final concentration
Penicillin	50-100 U/ml
Streptomycin sulfate	50-100 μg/ml
Kanamycin	100 μg/ml
Gentamycin	50 μg/ml
Neomycin	50 μg/ml
Mycostatin (Nystatin)	20 μg/ml
Amphotericin B (Fungizone)	0.25 μg/ml
Pen/strep/neomycin[a]	1×

[a]Usually supplied as 100× concentrated stock solution, which is diluted to 1× concentration in the medium.

as an adjunct to chemical disinfectant, does provide additional germicidal and viricidal control when the light is used and maintained properly (Meechan and Wilson, 2006).

We feel that a more reliable approach to eliminate contamination is the use of well-practiced microbiological procedures, good aseptic technique, and standard operational procedures for working in a biological safety cabinet, including thorough decontamination with an effective disinfectant before and after use of the biological safety cabinet. The current recommendation is that work surfaces be wiped down with ethanol instead of relying on UV lamps.

Frequently, specimens received in the laboratory are not sterile, and cultures prepared from these specimens may become contaminated with bacteria, fungus, yeast or mycoplasma. The presence of microorganisms can inhibit growth, kill cell cultures, or lead to inconsistencies in test results. The contaminants deplete nutrients in the medium and may produce substances that are toxic to cells. Antibiotics and antimycotics can be used to combat potential contaminants (see Table A.3F.1). The solutions can be used to wash specimens prior to culture or to rinse contaminated cultures, and can be added to medium used for tissue culture. Antibiotics (penicillin, streptomycin, kanamycin, neomycin, or gentamycin) can be used individually or in combinations (i.e., penicillin/streptomycin/neomycin and penicillin/streptomycin/gentamycin). Likewise, fungicides such as amphotericin B (Fungizone) and mycostatin (Nystatin) can be used alone or as antibiotic/antimycotic solutions (i.e., penicillin/streptomycin/Fungizone). Particular care should be taken when using Fungizone, as it is typically very toxic to cell cultures, and adequate data are not available regarding potential adverse effects on the growth of human cells. Antibiotics and antimycotics are available from a number of vendors including Life Technologies, Sigma-Aldrich, and BioWhittaker.

Cultures should be visually assessed on a routine basis for evidence of contamination (Coté, 1999b). Indicators in the tissue culture medium change color when contamination is present: for example, medium that contains phenol red changes to yellow because of increased acidity. Cloudiness and turbidity are also observed in contaminated cultures. Once contamination is confirmed with a microscope, infected cultures are generally discarded. Keeping contaminated cultures increases the risk of contaminating other cultures. Sometimes a contaminated cell line can be salvaged by treating it with various combinations of antibiotics and antimycotics in an attempt to eradicate the infection. In this procedure, the tissue culture medium is aspirated from the affected cultures and discarded. The cultures are rinsed with fresh "wash" medium that has been prepared by supplementing the routinely used tissue culture medium with the appropriate concentration of antibiotic and/or antimycotic (Table A.3F.1). The petri dish or flask should be

gently swirled so that the cell surface is bathed in the clean medium. The wash medium can be left on the cultures for 1 to 5 min. Due to the toxicity of Fungizone and amphotericin, it is recommended that cell exposure to these agents be limited to less than 2 min. Aspirate the wash medium, replace with fresh medium, and return the cultures to the incubator. Even under the best conditions, such treatment may adversely affect cell growth and it is often unsuccessful in ridding cultures of contaminants.

Mycoplasma are small (0.2 to 0.3 μm) intracellular bacteria that attach to the cell membrane, inhibit cell growth, and eventually lead to cell death. Because these parasites do not have cell walls, do not grow in colonies, and do not change the pH of the medium, they are difficult to detect visually in cultures. Mycoplasma can multiply to very high concentrations (10^7 to 10^8 organisms/ml) and adversely affect cultures by altering cell growth characteristics, inhibiting cell metabolism, disrupting nucleic acid synthesis, inducing chromosome aberrations, changing cell membrane antigenicity, and altering transfection rates and viral susceptibility. Mycoplasma are spread by cross-contamination from infected cultures through aerosolization during pipetting, or via the transfer of contaminated cells or contaminated reagents used in cell culture. Some laboratories require that cultures from other laboratories be tested for mycoplasma before they are accepted into the lab. There are several methods for detecting mycoplasma in cell cultures and in cell culture reagents (Coté, 1999b; Volokhov et al., 2011). In the direct method, both cultures and reagents can be plated on agar in order to grow and thereby reveal the presence of mycoplasma. Disadvantages of this method are that some strains of mycoplasma cannot be cultivated on agar and that the results can take from 2 to 4 weeks to obtain. Several indirect methods are available, and should be selected based on the capabilities of the laboratory and the needs of the laboratory in terms of sensitivity, specificity, and time requirements. The DNA staining method uses Hoechst 33258 to highlight the A-T rich DNA of mycoplasma, so that mycoplasma appear as bright extranuclear spots in the cytoplasm. Results are available in 24 hr, but this method is not as sensitive as the direct method and can be difficult to interpret due to background bacterial/yeast/fungal contamination, excess debris, reduced or absent live cells, and broken nuclei from dead cells. The addition of an indicator cell line such as Vero (Sigma-Aldrich) increases the sensitivity of this method. PCR testing that selectively amplifies part of the mycoplasma DNA provides rapid results and is highly sensitive. Testing kits and reagents are available from several suppliers including ATCC, Sigma-Aldrich, Agilent Technologies, Roche Applied Science, and PromoCell Gmbh. Enzymatic assay kits, which measure conversion of ADP to ATP in the presence of substrate, are another inexpensive and sensitive alternative testing format (Volokhov et al., 2011). Kits are available from suppliers such as Lonza. Lastly, another form of unique testing kit (InvivoGen) involves a visible color change in the testing medium when sensor cells are induced to secrete an enzyme in the presence of mycoplasma.

Good aseptic technique and routine testing, recommended after each cell passage, are the most effective methods for preventing mycoplasma contamination. Most standard antibiotics are not effective in treating mycoplasma contamination, and many laboratories prefer to dispose of contaminated cultures and any sterile reagents that were used with them. In the event that the cultures are valuable and backup cultures are not available, it is imperative to attempt to salvage the contaminated cells. All areas involved in cell culture, including the hood, incubator, and pipettors should be thoroughly cleaned and disinfected. Antibiotic reagents specifically for treatment of mycoplasma infections in cell cultures are available from multiple suppliers such as Sigma-Aldrich, Lonza, PromoCell Gmbh, and InvivoGen. For further information refer to Uphoff and Drexler (2014).

CULTURE MEDIUM PREPARATION

Choice of tissue culture medium comes from experience. An individual laboratory must select the medium that best suits the growth requirement of the type of cells being cultured while taking into account the goal of the particular experiment. Chemically defined media are available in liquid or powdered form from a number of suppliers. Sterile, ready-to-use medium has the advantage of being convenient, although it is more costly than other forms. Powdered medium must be reconstituted with tissue culture–grade water according to manufacturer's directions. Distilled or deionized water is not of sufficiently high quality for medium preparation; double- or triple-distilled water or commercially available tissue culture water should be used. The medium should be filter-sterilized and transferred to sterile bottles. Prepared medium can generally be stored $\leq$1 month in a 4°C refrigerator. Laboratories using large volumes of medium may choose to prepare their own medium from standard recipes. This may be an economical approach, but it is time-consuming and the savings may not offset the time required.

Basic media such as Eagle minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM; see recipe), Glasgow modified Eagle medium (GMEM), and RPMI 1640 and Ham F10 nutrient mixture (e.g., Life Technologies) are composed of amino acids, glucose, salts, vitamins, and other nutrients. A basic medium is supplemented by addition of L-glutamine, antibiotics (typically penicillin and streptomycin sulfate), and usually serum, to formulate a "complete medium." Where serum is added, the amount is indicated as a percentage of fetal bovine serum (FBS) or other serum. Some media are also supplemented with antimycotics, nonessential amino acids, various growth factors, and/or drugs that provide selective growth conditions. Supplements should be added to medium prior to sterilization or filtration, or added aseptically just before use.

The optimum pH for most mammalian cell cultures is 7.2 to 7.4. Adjust pH of the medium as necessary after all supplements are added. Buffers such as bicarbonate and HEPES are routinely used in tissue culture medium to prevent fluctuations in pH that might adversely affect cell growth. HEPES is especially useful in solutions used for procedures that do not take place in a controlled CO_2 environment.

Fetal bovine serum (FBS; sometimes known as fetal calf serum, FCS) is the most frequently used serum supplement. Calf serum, horse serum, and human serum are also used; some cell lines are maintained in serum-free medium (Freshney, 2010). Complete medium is supplemented with 5% to 30% (v/v) serum, depending on the requirements of the particular cell type being cultured. Serum is obtained frozen, then is thawed, divided into smaller portions, and refrozen until needed.

There is considerable lot-to-lot variation in FBS. Most suppliers will provide a sample of a specific lot and reserve a supply of that lot while the serum is tested for its suitability. The suitability of a serum lot depends upon the use. Frequently the ability of serum to promote cell growth equivalent to a laboratory standard is used to evaluate a serum lot. Once an acceptable lot is identified, enough of that lot should be purchased to meet the culture needs of the laboratory for an extended period of time.

All materials that come into direct contact with cultures must be sterile. Sterile disposable dishes, flasks, pipets, etc., can be obtained directly from manufacturers. Reusable glassware must be washed, rinsed thoroughly, and then sterilized by autoclaving or by dry heat before reusing. With dry heat, glassware should be heated 1.5 to 2 hr at 160°C to ensure sterility. Materials that may be damaged by very high temperatures can be autoclaved 20 min at 120°C and 15 psi. All media, reagents, and other solutions that come into contact with the cultures must also be sterile; media may be obtained as a sterile liquid from the manufacturer, autoclaved if not heat-sensitive, or filter sterilized.

Supplements can be added to media prior to filtration, or they can be added aseptically after filtration. Filters with 0.20- to 0.22-μm pore size should be used to remove small Gram-negative bacteria from culture media and solutions.

Although the use of heat-inactivated serum was once preferred, it is no longer standard, or may be standard in some labs simply because it is an established and routine practice. Historically, heat inactivation was considered necessary to destroy complement protein due to its role in cell lysis. It has since been shown that the level of complement components in commercially available FBS was only 1% to 3% of adult levels, and that no significant cell lysis was detected with the use of commercially available FBS, even when undiluted (Triglia and Linscott, 1980). In addition, many researchers pre-warm FBS to 37°C, which is enough to inactivate heat-labile complement components. Improvements in the filtration of serum products have also made the use of heat-inactivated serum nonessential for most cell types. In the past, serum was filtered through 0.45-μm or 0.22-μm filters, raising concern that adventitious agents such as mycoplasma could persist. Today, media suppliers use 0.1-μm or 0.04-μm porosity membranes to eliminate the need for heat inactivation. Not only is heat inactivation unnecessary for most cell types, it may sometimes be detrimental to cell growth. Heat inactivation not only destroys complement, but also degrades amino acids, vitamins, growth factors, and other nutrients that enhance cell growth. Furthermore, heat inactivation can increase the formation of precipitates that can be mistaken for microbial contamination. Laboratories that continue the use of heat inactivation should conduct studies to determine whether this step is really indicated. Heat inactivation may be warranted when conducting immunologic studies or when culturing embryonic stem cells, insect cells, and smooth muscle cells. For cells obtained from a commercial vendor such as ATCC, serum requirements are included in the cell descriptions.

Although FBS has historically been the serum of choice, many investigators are moving towards the use of alternative types of animal serum or to serum-free media. An increasing number of companies are offering serum-free media for a wide range of cell types. Although the term "serum-free" implies that a medium contains no serum, the medium may not be entirely free of serum-derived products. For example, bovine serum albumin may be used as the protein component of particular types of serum-free media (Newman, 2003). While some media are designed for culturing a particular cell type, others are general-purpose media that can support a variety of cell types.

Commercially prepared media containing L-glutamine are available, but many laboratories choose to obtain medium without L-glutamine, and then add it to a final concentration of 2 mM just before use. L-Glutamine is an unstable amino acid that, upon storage, converts to a form cells cannot use. Breakdown of L-glutamine is temperature- and pH-dependent. To prevent degradation, 100× L-glutamine should be stored frozen in aliquots until needed. Another option to prevent degradation is the use of a stabilized dipeptide form (L-glutamine or L-alanyl-L-glutamine). Sources for this include ATCC, Life Technologies, Merck Millipore, and Corning Cellgro.

In addition to practicing good aseptic technique, most laboratories add antimicrobial agents to medium to further reduce the risk of contamination. A combination of penicillin and streptomycin is the most commonly used antibiotic additive; kanamycin and gentamicin are used alone. Mycostatin and amphotericin B are the most commonly used fungicides (Rooney, 2001). Table A.3F.1 lists the final concentrations for the most commonly used antibiotics and antimycotics. Combining antibiotics in tissue culture media can be tricky, as some antibiotics are not compatible and one may inhibit the action of another. Furthermore, combined antibiotics may be cytotoxic at lower concentrations than the individual antibiotics. In addition, prolonged use of antibiotics may cause cell

lines to develop antibiotic resistance. For this reason, some laboratories add antibiotics and/or fungicides to medium when initially establishing a culture but eliminate them from medium used in later subcultures.

Commercially available tissue culture media have been tested for sterility prior to release, and further testing within the research laboratory is generally not required. In clinical laboratories, sterility checks on tissue culture media are often performed as a quality-control monitor in compliance with requirements from the College of American Pathologists (CAP). A small aliquot from each lot of medium is incubated 48 hr at 37°C and monitored for evidence of contamination such as turbidity (infected medium will be cloudy) and color change (if phenol red is the indicator, infected medium will turn yellow). Any contaminated medium should be discarded.

CONTINUOUS CELL LINES

Immortal, or continuous, cell lines are those that have the ability to divide indefinitely. Continuous cell lines have the advantage of allowing a researcher to use the same line for long-term studies that may proceed over decades. Other advantages of using a continuous cell line are that they are homogeneous, can be well characterized, are easy to maintain, and propagate readily. A disadvantage is that they have been altered to divide indefinitely in an artificial environment; thus, they are not representative of in vivo conditions. In contrast to continuous cultures, primary cultures are heterogeneous, are not well characterized, have a limited lifespan, and proliferate slowly. The more times primary cells are passaged, the more their characteristics change. Nonetheless, primary cultures are felt to better represent the in vivo condition, particularly if used in early passages when they are less like to have undergone changes attributed to genetic drift. Some investigators prefer to use cell lines for their preliminary research and primary cells for confirmatory studies. The goal of the research project will dictate the type of cells utilized.

There are many options when it comes to using immortal cell lines. Some investigators choose to purchase well-characterized cell lines from a commercial source [e.g., ATCC, Life Technologies, Coriell Cell Repository, European Collection of Cell Culture (ECACC)] or from a trusted colleague, while others prefer to establish their own cell lines. Transfection of primary cultures with a retrovirus vector containing an immortalizing gene can result in deregulation of the cell cycle by blocking inhibition of cell-cycle progression. This results in an increased life span, but also leads to reduced DNA surveillance, thereby affording the opportunity for further mutations (Freshney, 2010). This method may be problematic as the resulting cell line may be heterogeneous. Protocols for immortalization of fibroblasts by SV-40 transfection and by telomerase induction are provided by Freshney (2010). Lymphoblastoid cell lines derived from peripheral blood provide a constant source of material obtained by a simple blood draw. For a review of the advantages and drawbacks of using lymphoblastoid cells lines, along with steps required to establish lymphoblastoid cell lines, refer to the review by Hussain and Mulherkar (2012).

Cancer researchers may elect to establish cell lines from tumors, particularly those that are aggressively growing. The three volumes by (Masters and Palsson 2002a, 2002b, 2002c) provide a comprehensive guide for culturing cells from a variety of tumor types.

ESTABLISHING PRIMARY CULTURES FROM TISSUE

BASIC PROTOCOL 1

In order to provide cells with free access to the culture surface, cells from tissue may be dispersed by fine mincing followed by enzymatic treatment. Enzymatic dispersal is performed by treating the minced tissue fragments with a collagenase solution. Collagenase treatment will cause individual cells to dissociate from the fragments. Viable cells will

attach to the culture surface and begin dividing. Minced tissue, or explants, may also be placed directly into culture without collagenase treatment. Cells will grow outward onto the culture vessel surface. The culture method should be matched to the tissue type and how the cultured cells will be used. For example, it may be desirable to study the cells in situ. In this case, growing the cells on cover slips in Petri dishes may be preferable to growing them in flasks. When large numbers of cells are required, flask cultures are recommended. Additionally, flasks, unlike cultures grown in Petri dishes, may be grown in either closed or open (5% CO_2 environment) incubators. The following protocol was developed for human solid tissue sources (Priest, 1997).

The tissue specimen should be obtained under sterile conditions and transported in sterile culture medium, Hanks balanced salt solution (HBSS), or phosphate-buffered saline (PBS). It is important that the tissue not become dry. Cultures should be initiated within 24 hr of tissue sampling to maintain cell viability. If cultures cannot be initiated immediately, it is advisable to store the tissue at 4°C. Cultures cannot be initiated from tissue that has been placed in formalin.

Materials

Fresh tissue
Complete RPMI (see recipe)
Complete RPMI with Fungizone (optional; recommended if tissue was not collected aseptically; see recipe)
Complete α-MEM medium (see recipe)
Collagenase Type I, lyophilized, from *Clostridium histolyticum*, activity greater than 125 U/mg (Life Technologies/Gibco or other source)

Sterile petri dishes: 60 × 15 mm or 100 × 15 mm
Sterile forceps
Sterile plastic serological pipets (1, 5, and 10 ml)
Disposable safety scalpels
15-ml conical centrifuge tubes (e.g., BD Falcon)
10-ml sterile syringe with 21-G needle (Luer-lok tip and blunt-fill needle)
Nalgene syringe filter (0.2 μm)
Clinical centrifuge
25-cm^2 (T-25) culture flasks
Inverted microscope

NOTE: This is an aseptic procedure. All precautions must be taken to ensure the protection of the technologist and to maintain sterility of the specimen and reagents. Protective clothing must be worn at all times when handling specimens.

1. *Optional:* At this point, if the tissue was not collected under aseptic conditions, soak the tissue for several hours or overnight in complete RPMI with Fungizone to minimize potential contamination of cultures. For tissue obtained in a sterile environment, go to step 2.

2. Transfer tissue to a sterile 60 × 15 mm or 100 × 15 mm sterile Petri, depending on the amount of specimen received. If specimen is very large, excise several small sections with scalpel and forceps, then put into a 60 × 15 mm sterile Petri dish. Add sufficient complete RPMI medium to keep specimen moist.

3. Place approximately 5 ml of complete RPMI (without Fungizone) into a second sterile 60 × 15 mm petri dish.

   This will be used to wash the tissue. More Petri dishes with RPMI may be needed to successively wash very bloody tissues.

4. Using a sterile scalpel and forceps, cut off enough of the original tissue to be able to set up at least 4 flasks (or, if using in situ cultures, for 8 coverslip cultures).

   A piece approximately 5 cm^3 treated with collagenase will yield sufficient cells to set up two to three 25-cm^2 (T-25) flasks. Avoid very bloody portions or wash (by swishing around dish with forceps) away blood with medium. If needed, transfer to fresh petri dishes with RPMI medium until tissue is free of blood. Scrape away any adhering clots. Some tissue may need to be removed if it is not of the desired type. Most viable tissue will be pink, white, or tan in color. Tissue which is yellow and soft or gray is likely to be necrotic.

5. Once the tissue has been cleaned, finely minced each large piece in a fresh petri dish with medium, using sterile forceps and scalpel.

   This can be performed in more than one dish.

6. Divide the contents of each dish (liquid plus tissue) evenly into two, 15-ml sterile conical centrifuge tubes. This can be done using a 10-ml pipet. Label each centrifuge tube with the specimen ID number and, if two α-MEM medium lot numbers are going to be used, the tubes should be marked accordingly. Lightly cap the tubes and place in rack.

7. Determine the amount of collagenase needed.

   For the next step, collagenase is weighed and dissolved in complete RPMI medium. The final concentration of collagenase that will be needed is 1 mg/ml in each of the two tubes prepared in step 6. In general, the amount needed is equal to twice the volume of the sum of the two tubes. The amount of collagenase needed is dependent upon the volumes already in the two tubes with minced tissue and the volume of medium that is needed to dissolve the collagenase.

   Example: After tissue mincing and transfer to the two tubes, one tube contains 2.5 ml, and the other tube contains 3.5 ml (total 6 ml). Based upon doubling that volume, you would weigh out 12 mg collagenase and dissolve it in 6 ml of RPMI medium in a clean Petri dish.

8. Weigh out the amount of collagenase needed. Carefully transfer the collagenase into another sterile petri dish containing the amount of complete RPMI calculated above (in the example above, this would be 6 ml). Allow the collagenase to dissolve for a few seconds and then aspirate the collagenase solution into a 10-ml syringe.

9. Remove the needle from the syringe and attach a 0.2-μm syringe filter to the barrel. Into each centrifuge tube containing the minced tissue, carefully dispense (through the filter) a volume of fluid equal to the volume already in each centrifuge tube.

10. Re-cap the tubes and incubate at 37°C for approximately 30 min. Shake the tubes to check for cloudiness every 10 min, which indicates the collagenase is working.

    The suspension should be cloudy but not to the point where there are no intact pieces of tissue left.

11. Add ~5 ml complete α-MEM to the each tube (if using two lots of medium, make sure that the tubes are labeled accordingly).

    This addition of complete medium will stop the action of the collagenase.

12. Centrifuge 9 to 10 min at 1000 rpm in a clinical centrifuge.

13. Label two 25-cm^2 (T-25) culture flasks for each centrifuge tube that has been prepared. Label all flasks with the specimen identifiers and a culture designation (1, 2, 3 . . . or A, B, C . . .).

14. After specimens are centrifuged, remove supernatant with a sterile pipet, leaving approximately 2 ml of tissue suspension. Add fresh complete α-MEM to bring the volume in each tube up to 4 ml. Resuspend the cell mixture. With a sterile pipet, place approximately 2 ml of the suspension into each of the two flasks with the flask lying horizontally. Try to evenly distribute any larger tissue pieces between the flasks. It may be necessary to add more fresh medium to just cover the bottom of the flask. Tissue pieces should not be floating in medium.

15. Incubate all flasks at 37°C in a humidified 5% CO_2 incubator in a horizontal position with caps slightly loosened. Record when cultures were established. If two lot numbers of medium are used, record which lot number is in each flask.

16. Monitor flasks with an inverted microscope for evidence of cell attachment beginning on Day 2 after culture initiation.

    Caps on flasks should be tightened when cultures are removed from the incubator for this purpose. When cell growth is observed 2 to 3 ml of α-MEM (using same lot number as before) is added. Flasks that do not show evidence of cell attachment and growth should be left undisturbed for at least 5 days; however, make sure that there is still medium covering the bottom of the flask (cells must NOT dry out!). Remember to loosen caps again before returning the cultures to incubator. Record the date of each observation, what was observed, and what action was taken.

17. Perform a complete change of medium once cell attachment and growth is noted by pipetting off the old medium while gently slanting the flask. Add 5 ml fresh α-MEM (same lot number), loosely cap the flask, and place in horizontal position in incubator as before. Record when the change of medium takes place.

18. Perform subsequent medium changes as needed every 3 to 5 days.

    Rapidly growing cultures will require more frequent medium changes.

TRYPSINIZING AND SUBCULTURING CELLS FROM A MONOLAYER

BASIC PROTOCOL 2

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 (Basic Protocol 1)
HBSS without Ca^{2+} and Mg^{2+} (e.g., Life Technologies), 37°C
0.25% (w/v) trypsin/0.2% EDTA solution (see recipe), 37°C
Complete medium with serum: e.g., DMEM supplemented with 10% to 15% (v/v) fetal bovine serum (complete DMEM-10; see recipe), 37°C

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 incubations are 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 (Support Protocol 3) 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 petri plates or flasks (e.g., 150-mm plates or 75-cm^2 flasks) may be used for later subcultures.

   Cultures dishes/flasks should be labeled with at least two unique identifiers, as well as date of culture 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.

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.

ALTERNATE PROTOCOL 1

PASSAGING CELLS IN SUSPENSION CULTURE

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 incubations are 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.

For materials, see Basic Protocol 2.

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 the 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 an 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 by swirling flask to resuspend cells and observing color changes from pink to yellow/orange in the medium, which indicate active cell metabolism.

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, retaining the other half of the cell suspension in the original flask.

   Alternatively, the entire cell suspension can be removed from the original flask and divided equally into two fresh flasks. The original flask can be discarded, or if there is concern about the need for additional cells, the original flask can be retained and fed in an attempt to salvage any residual cells.

   c. Feed each flask with 7 to 10 ml prewarmed medium and return flasks 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 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 agent 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

Freezing medium: complete medium (e.g., DMEM or RPMI; see recipes) supplemented with 10% to 20% (v/v) FBS and 5% to 10% (v/v) DMSO, 4°C
Benchtop clinical centrifuge with 45° fixed-angle or swinging-bucket rotor

NOTE: All incubations are 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. Trypsinize cells from plate (see Basic Protocol 2, 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*, room temperature.

   The benchtop centrifuge can be accessorized depending on the anticipated volume. A variety of brands are available, including Eppendorf, Thermo, and Beckman, and can be obtained from Fisher Scientific, VWR, and other laboratory equipment vendors.

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

3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet to obtain a density of 1×10^6 cells/ml.

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 roughly 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 a liquid nitrogen storage freezer.

   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.

For materials, see Support Protocol 1.

NOTE: All incubations are 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. Transfer cell suspension to a centrifuge tube and spin 10 min at 300 to 350 × *g*, 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 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.

NOTE: All incubations are 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.

Materials

Cryopreserved cells stored in liquid nitrogen freezer
70% (v/v) ethanol
Complete medium (e.g., DMEM or RPMI; see recipes) containing 10% to 20% FBS (see recipe), 37°C

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 containing 20% FBS. Centrifuge 10 min at 150 to 200 × *g*, 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 a 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 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 extra careful of this when working with cell lines known to be mosaic.

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

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

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from VWR (Fig. A.3F.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

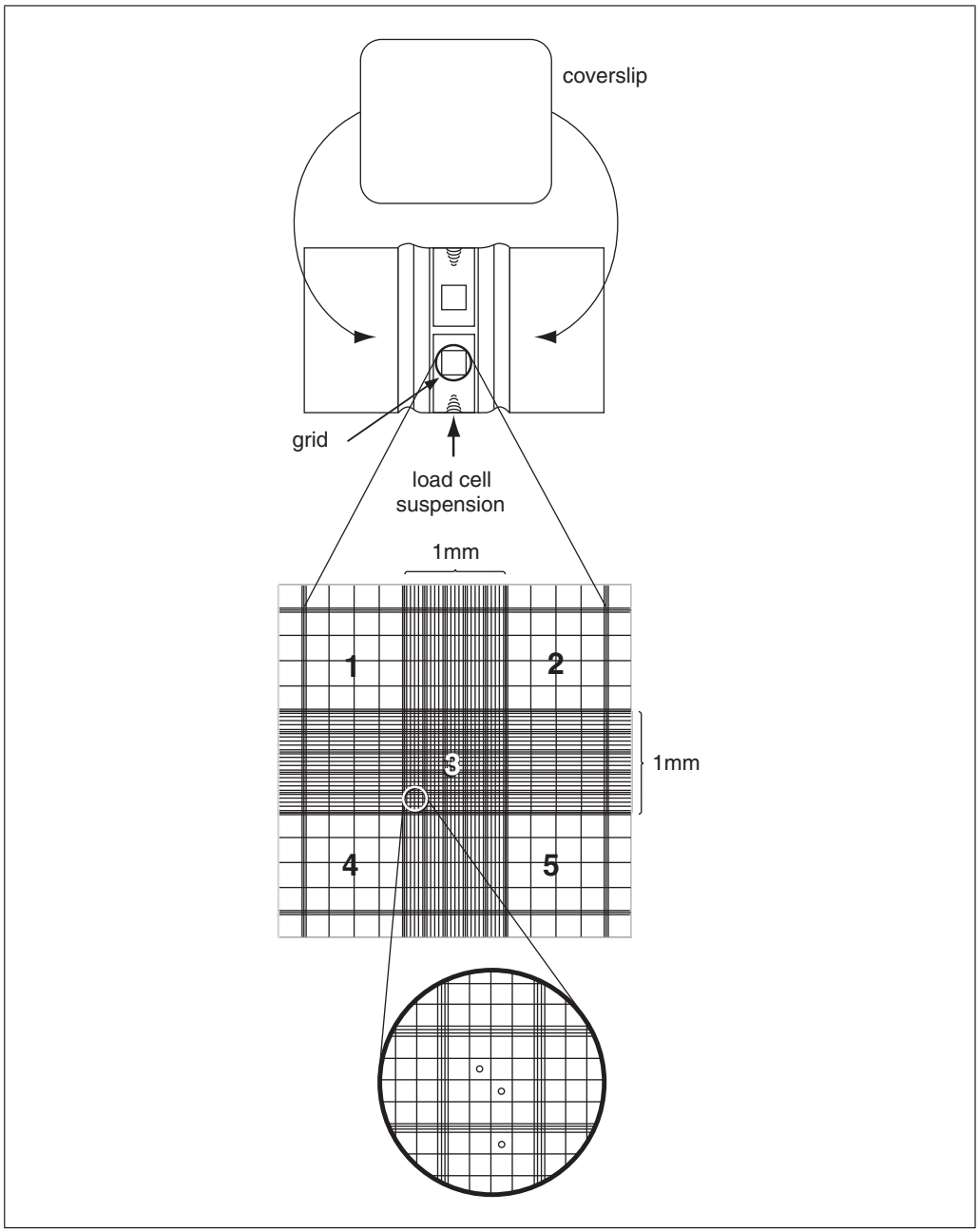

Figure A.3F.1 Hemacytometer slide (Improved Neubauer) and coverslip. Coverslip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipet. Each counting chamber has a 3 × 3-mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted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

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, which can introduce errors in cell counting; therefore, it is imperative that the coverslip provided with the hemacytometer be used in determining cell number.

Materials

70% (v/v) ethanol
Cell suspension
0.4% (w/v) trypan blue or 0.4% (w/v) nigrosin, prepared in HBSS

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

NOTE: A disposable plastic hemacytometer, the INCYTO C-Chip, has exactly the same grid pattern as the Improved Neubauer. It is a single-use device available through several distributors.

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 so the coverslip rests 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 2).

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-mm square is recommended.

Load hemacytometer

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

   Suspension will be drawn under the coverslip by capillary action.

   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. A.3F.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. A.3F.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 ml 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 volume correction factor for the hemacytometer is 10^4: 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, whereas live cells will be impermeable to it.

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

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

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.* It is also imperative that the flasks have non-vented caps. 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, distilled water in all recipes and protocol steps. Suitable suppliers for media and components include Life Technologies/Gibco, Cambrex, Corning, Hyclone, and Sigma-Aldrich.

Complete α-MEM Medium

α-MEM with Earle's Salts (Irvine Scientific or other source, preferably two different lot numbers) supplemented with:
20% fetal bovine serum
100 U/ml penicillin/100 μg/ml streptomycin sulfate (add from 100 × pen/strep stock; Sigma Aldrich)
2 mM L-glutamine (add from 100× stock; see recipe)

Prepare the above using aseptic technique. Store complete medium at 4°C; use within 14 days

IMPORTANT NOTE: *Medium must be previously tested for sterility and ability to support growth.*

Complete DMEM

Dulbecco's modified Eagle medium, high-glucose formulation (e.g., Invitrogen), containing:
5%, 10%, or 20% (v/v) FBS (optional; see recipe)
1% (v/v) nonessential amino acids
2 mM L-glutamine (see recipe)
100 U/ml penicillin/100 μg/ml streptomycin sulfate (add from 100 × pen/strep stock; Sigma Aldrich)
Filter sterilize and store ≤1 month at 4°C

Throughout this manual, the percentage of serum (usually fetal bovine serum) used in a protocol step is indicated by a numeral hyphenated to the base medium name. Thus, "complete DMEM-10" indicates that 10% FBS is used. Absence of a numeral indicates that no serum is used.

DMEM containing 4500 mg/liter D-glucose can be obtained from Life Technologies.

DMEM is also known as Dulbecco's minimum essential medium.

Fetal bovine serum (FBS)

Thaw purchased fetal bovine serum (shipped on dry ice and kept frozen at −20°C until needed). Store 3 to 4 weeks at 4°C. If FBS is not to be used within this time, aseptically divide into smaller aliquots and refreeze until used. Store ≤1 year at −20°C.

Repeated thawing and refreezing should be avoided as it may cause denaturation of the serum.

In some cases, heat inactivation may be warranted (see Culture Medium Preparation at the beginning of this appendix). To inactivate FBS, heat 30 to 60 min in a 56°C water bath. Alternatively, FBS may be inactivated through radiation treatment.

L-Glutamine, 0.2 M (100×)

Thaw frozen L-glutamine, aliquot aseptically into usable portions, then refreeze. For convenience, L-glutamine can be stored in 1-ml aliquots if 100-ml bottles of medium are used, and in 5-ml aliquots if 500-ml bottles are used. Store ≤1 year at −20°C.

Many laboratories supplement medium with 2 mM L-glutamine—1% (v/v) of 100× stock—just prior to use.

Complete RPMI

RPMI 1640 medium (e.g., Life Technologies), supplemented with:
2%, 5%, 10%, 15%, or 20% (v/v) FBS (optional, see recipe)
2 mM L-glutamine (see recipe)
100 U/ml penicillin/100 μg/ml streptomycin sulfate (add from 100 × pen/strep stock; Sigma Aldrich)
Filter sterilize and store 1 month at 4°C

Complete RPMI with Fungizone

Add 0.5 ml of Fungizone (Life Technologies; 250 μg/ml Amphotericin B) to 100 ml complete medium (see recipe). Store up to 2 months at 2° to 4°C or up to 3 months at −5° to 20°C, or up to the expiration date for the medium.

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

Most laboratories prefer to purchase trypsin/EDTA as a prepared solution, which is available from vendors including Sigma-Aldrich, Life Technologies, and Cambrex. This is a convenient and cost-effective alternative to preparing the solution within the laboratory. 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 aliquotted into smaller volumes. Specific applications may require different concentrations of trypsin; the appropriate methods should be consulted for details.

An alternative to traditional trypsin/EDTA is TrypLE trypsin from Life Technologies. It is stable at room temperature, does not require inactivation by an inhibitor, and is gentle on cells, which results in high viability rates.

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, 2010). Cells dispersed from the original tissue can be grown in monolayers and passaged repeatedly to give rise to a relatively stable cell line. Some types of cells can be grown in suspension cultures.

Four distinct growth stages have been described for primary cells maintained in culture. First, cells adapt to the in vitro environment in a lag phase. Second, cells undergo an exponential or log phase growth lasting through ~30 passages. Third, the growth rate of cells slows in the stationary phase, leading to a progressively longer generation time. Finally, after 40 or 50 passages, cells begin to senesce and die. It may be desirable to study a particular cell line over several months or years, so monolayer 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 monolayer cultures prevents changes due to genetic drift and avoids loss of cultures due to senescence or accidental contamination (Freshney, 2010).

In the United States, cell lines are commercially available from a number of sources, including the American Type Culture Collection (ATCC; *http://www.atcc.org*) and the Human Genetic Mutant Cell Repository at the Coriell Cell Repository (CCR; *http://www.coriell.org*). The European Collection of Cell

Cultures (ECACC; *http://www.phe-culturecollections.org.uk*) and CellBank Australia (*http://www.cellbankaustralia.com*) are just two examples of other resources for research cell lines. These cell repositories are a valuable resource for researchers who do not have access to suitable patient populations. The cell lines are preserved in liquid nitrogen, and information on the characteristics are supplied by the distributor. Immortalized cell lines can be maintained in culture indefinitely, while nontransformed cells have a limited life-span in vitro. For human fibroblasts, about 20 population doublings occur between the initial growth in the primary culture and the first subculture (Priest, 1997). About 50 to 60 population doublings is the standard rule for maintaining fibroblast cell lines in culture before risking the loss of cell lines through senescence, cytogenetic or biochemical changes, or contamination. If it is anticipated that these cells will be needed for future study, cells should be frozen at an early culture stage and retrieved for future use.

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 monolayer cultures 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 $\sim 5 \times 10^4$ cells/ml in each new culture of human cell lines. Optimal concentrations are cell-type specific. Package inserts provided with commercially available cell lines contain information on recommended cell density, culture media, and appropriate culture temperatures. 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, 2010).

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 avoid potential 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 monolayer culture is directly dependent on the available surface area of the culture vessel (Freshney, 2010). 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 over 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 cells. Cultures should be inspected regularly for signs of contamination and to determine if the culture needs feeding or passaging.

Literature Cited

Burgener, J. 2006. Position paper on the use of ultraviolet lights in biological safety cabinets. *Appl. Biosaf.* 11:228-230.

Coté, R.J. 1998. Aseptic technique for cell culture. *Curr. Protoc. Cell Biol.* 00:1.3.1-1.3.10. doi: 10.1002/0471143030.cb0103s00.

Coté, R.J. 1999a. Sterilization and filtration. *Curr. Protoc. Cell Biol.* 1:1.4.1-1.4.21. doi: 10.1002/0471143030.cb0104s01.

Coté, R. 1999b. Assessing and controlling microbial contamination in cell cultures. *Curr. Protoc. Cell Biol.* 1:1.5.1-1.5.18. doi: 10.1002/0471143030.cb0105s01.

Freshney, R.I. 2010. Culture of Animal Cells. A Manual of Basic Technique and Specialized Applications, 6th ed. Wiley-Blackwell, New York.

Hussain, T. and Mulherkar, R. 2012. Lymphoblastoid cell lines: A continuous in vitro source of cells to study carcinogen sensitivity and DNA repair. *Int. J. Mol. Cell Med.* 1:75-87.

Masters, J.R.W. and Palsson, B. (eds.) 2002a. Human Cell Culture Vol I Cancer Cell Lines Part 1. Kluwer Academic Publishers, New York.

Masters, J.R.W. and Palsson, B. (eds.) 2002b. Human Cell Culture Vol II Cancer Cell Lines Part 2. Kluwer Academic Publishers, New York.

Masters, J.R.W. and Palsson, B. (eds.) 2002c. Human Cell Culture Vol III Cancer Cell Lines Part 3: Leukemia and Lymphoma. Kluwer Academic Publishers, New York.

Meechan, P.J. and Wilson, C. 2006. Use of ultraviolet lights in biological safety cabinets: A contrarian view. *Appl. Biosaf.* 11:222-227.

Newman, C. 2003. Serum-free cell culture—the ethical, scientific and economic choice. *Biomed. Sci.* 941-942.

Priest, J.H. 1997. General cell culture principles and fibroblast culture. *In* The AGT Cytogenetics Laboratory Manual, 3rd ed. (M.J. Barch, T. Knutsen, and J.L. Spurbeck, eds.) pp. 173-197. Lippincott-Raven, Philadelphia.

Rooney, D.E. (ed.) 2001. Human Cytogenetics: Constitutional Analysis: A Practical Approach, 3rd ed. Oxford University Press, New York.

Triglia, R.P. and Linscott, W.D. 1980. Titers of nine complement components, conglutinin and C3b inactivator in adult and fetal bovine sera. *Mol. Immunol.* 17:741-748. doi: 10.1016/0161-5890(80)90144-3.

U.S. Department of Health and Human Services. 2009. Centers for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories, 5th ed. HHS Publication No. (CDC) 21-1112. Governmental Printing Office, Washington D.C. Available at *http://cdc.gov/biosafety/publications/bmbl5/BMBL.pdf*.

Uphoff, C. C. and Drexler, H. G. 2014. Detection of mycoplasma contamination in cell cultures. *Curr. Protoc. Mol. Biol.* 106:28.4.1-28.4.14.

Volokhov, D.V., Graham, L.J., Brorson, K.A., and Chizhikov, V.E. 2011. Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. *Mol. Cell. Probes* 25:69-77. doi: 10.1016/j.mcp.2011.01.002.

Key Reference

Freshney, R.I. 2010. See above.

Contains pertinent information on cell culture requirements and techniques for many tissue types.

Internet Resources

http://www.atcc.org

The Web site of the American Type Culture Collection, a non-profit biological resource center, has an excellent document library for various cell culture topics.

http://www.coriell.org

The Web site for the Coriell Institute for Medical Research.

http://www.lifetechnologies.com/us/en/home/technical-resources/technical-reference-library.html

This Web site has documents about general cell culture, protocols, and troubleshooting.

http://www.lifescience.roche.com

The document resources section of the Roche Life Science site contains a technical guide for culture of animal cells.

http://www.phe-culturecollections.org.uk

The Web site for Public Health England includes four separate collections of cell lines and microbial strains. A free handbook on cell culture is available in the section for the European Collection of Cell Cultures (ECACC).