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Essential Techniques of Cancer Cell Culture

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1. Introduction

Cell culture utilizes a number of core techniques, and although there can be marked diversity in how these procedures are practiced, there are elements and features that are universally applied. This chapter describes some of the essential techniques and provides typical protocols. It is assumed that aseptic technique will be used for all these procedures unless mentioned otherwise.

1.1. Primary Culture

A primary cell culture is the initial culture set up directly from a body tissue. Primary cancer cultures can be initiated and derived from a variety of tissue types such as solid tumor fragments (primary or metastatic) or cell suspensions, for example, aspirates, including peritoneal ascites or pleural effusions. Cell suspensions can be particularly convenient for developing cell lines as they are already growing as single cells or clusters, avoiding the need for mechanical or enzymatic dispersion. The cellular composition of primary cultures is often very variable with hematopoietic and stromal cell types contributing to the cellular mix. Fibroblasts, in particular, can be problematic as they attach readily to matrices and often outgrow the cancer cell population. Cancer cells differ from most normal cell types in their ability to grow in suspension, for example, in agar, but generally cultures are initiated by allowing cells to adhere to a substrate before proliferating. A number of strategies have been developed to help disperse fragments of tissue and these include mechanical and enzymatic methods *(see* **Subheading 1.2.)**.

1.2. Routine Feeding and Maintenance

Cell cultures should be examined regularly and routinely (preferably daily) both macroscopically and microscopically. The cell morphology and cell density should be checked by microscope and the presence of contaminants such as fungus and bacteria should be evaluated. Macroscopically, the color and turbidity of the medium should be monitored. Media (plus serum and other additives) should be changed regu-

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larly and not allowed to become depleted, depriving cells of specific nutrients, or becoming acidic. The pH of the medium is most easily monitored by the addition of color indicators such as phenol red. The frequency of media renewal will be dependent on the growth rate of the culture with more rapidly growing cultures requiring more regular changes.

1.3. Subculture of Cells

When a culture has occupied the complete surface of a flask (for a monolayer culture) or has grown to a point where media has been depleted of nutrients (for a suspension culture), then it requires "subculture" (also described as "passaging" or "splitting") to maintain healthy growth. This process reduces the cell density back to a level where the cells will grow optimally again and not exhaust the medium of nutrients too rapidly. The process of detachment of the adherent monolayer to give a single cell suspension is often referred to as "harvesting." The use of proteolytic enzymes such as trypsin, breaks the cell-cell and cell-substrate links and creates a single-cell suspension. Subculture of a primary culture into a secondary culture produces a "cell line." Once a cell-line is created, not only should it be given a designation, but a record of the number of subcultures or passages should be kept to provide a general indication of the lifetime of the culture. The cell line should be characterized and this process is described in Part II.

1.4. Cloning

A clone is the population of cells derived from an individual cell and cloning is the process of isolating this individual cell and developing its progeny. Cloning will thus produce cultures that are genetically homogeneous at the outset. The colony forming efficiency (CFE) is a measure of the ability of a culture or cell line to produce colonies and represents the number of colonies produced/number of individual cells cultured. Primary cultures tend to have low CFE values, typically <1% while cell lines generally have much higher values, typically varying from 10–100%. Methods describing the cloning of cells on plastic and within agar are described.

1.5. Cell Counting

The determination of cell number is a key measurement both for setting up experiments with cancer cell lines as well as monitoring cell responses under experimental conditions. Two protocols provided here have advantages depending on the application, for example, the scale of the experiment or the number of cell lines to be counted. The simplest protocol involves the use of a hemocytometer (Improved-Neubauer) and is appropriate when only a small number of samples are to be counted. This is the least expensive approach because minimal outlay on equipment or reagents is required. A hemocytometer is an etched glass chamber that will hold a quartz cover slip exactly 0.1 mm above the chamber floor.The counting chamber is precisely etched in a total surface area of 9 mm^2. Calculation of cell number is based on counting the number of cells within a defined area underneath the cover slip. The second method is automated and involves the use of an electronic counter such as the Z2 from Beckman Coulter. This approach allows rapid and accurate counting of large numbers of cultured cells

and is widely used within the biomedical sciences but involves a greater initial outlay for the equipment.

1.6. Cryopreservation

The preservation of cell stocks at temperatures below –130°C has allowed the long-term storage of cells for periods of at least 2–3 decades. Several features are important for optimizing the viability of cells. The use of cryoprotective agents that prevent ice crystals forming and the fragmenting of membranes is essential. The most commonly used cryoprotective agent is dimethylsulfoxide (DMSO), but glycerol is an alternative. The rates of freezing and thawing also influence viability, and a freezing rate of approx 1°C/min is considered optimal. In contrast, thawing should be rapid and this is most easily achieved by placing ampules in a water bath at 37°C. Cells are generally stored in liquid nitrogen at –196°C, but can remain viable for short periods of time at –80°C.

1.7. Troubleshooting

A number of simple problems are routinely encountered in cell culture studies. Some of the more common issues are listed in **Table 1** with potential causes and their associated solutions.

2. Materials

2.1. Primary Culture

1. Specimen collection containers, for example, sterile Universal containers.
2. Cell disaggregation enzymes.
3. Sterile scissors.
4. Sterile forceps.
5. Sterile scalpels.
6. Tissue sieve.
7. Phosphate buffered saline (PBS).
8. Cell culture medium, for example, Dulbecco's modified Eagle's medium (DMEM), + 10% fetal calf serum (FCS), penicillin (100 U/mL)/streptomycin (100 mg/mL).

2.2. Routine Maintenance and Feeding

1. Cell culture media.
2. Inverted microscopes.
3. Trypsin-EDTA (for adherent cultures).
4. Phosphate-buffered saline (PBS) for adherent cultures.
5. Tissue culture plastics.

2.3. Subculture

1. Any adherent cell culture, for example, SKOV-3 ovarian cancer cell line.
2. Tissue culture flasks.
3. Complete cell culture medium, for example, RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS).
4. Trypsin–EDTA (Gibco BRL).
5. Dulbecco's PBS (Oxoid, Unipath Ltd, Basingstoke, England).

Table 1
Common Problems Associated With Cell Culture

Problem	Possible cause	Potential solution
Cells difficult to remove from plastic	Enzyme solution too weak	Higher concentration needed
	Inhibitor present in medium (for example, serum)	Cells require more careful washing
	Cells too confluent and enzyme cannot access cell-substrate interface	Cells require trypsinisation at lower cell density
Cells not adhering readily to plastic	Cells too heavily treated with trypsin	Use less trypsin or treat for less time
	Insufficient serum or attachment factors	Add more
	Dissociating agent (for example, not inactivated fully)	Add serum or specific inhibitors
	Mycoplasma contamination	Discard if infected
Suspension cells clumping together	Mycoplasma contamination	Discard if infected
	DNA from lysed cells sticking cells together	Add DNAse
Poor growth in culture	Absence or lower than normal levels of certain additives	Add missing components
	Contamination by bacteria, mycoplasma or fungi	Discard if infected
	Cell density too low	Increase density
Cell death/low viability	Incorrect pH	Correct pH
	Faulty media	Correct preparation
Too acidic pH	CO_2 content too high	Modify
	Contamination	If infected, discard
Too basic pH	Insufficient CO2	Caps too tight
	Too few cells	Increase cell density

2.4. Cloning

1. Cells.
2. Cell culture medium.
3. Trypsin-EDTA.
4. Hemocytometer or cell counter.
5. Petri dishes.
6. Agar.

2.5. Cell Counting

2.5.1. Hemocytometer Counting

1. Cell line of interest.
2. PBS.
3. Trypsin-EDTA.
4. Tissue culture media containing 10% FCS.
5. Tally Counter.
6. Syringe and needles or Pasteur pipets.
7. Microscope.
8. Hemocytometer (Improved Neubauer).
9. 0.4% Trypan blue in PBS (optional).
10. 10% Formalin (optional).

2.5.2. Electronic Counting

1. As above but with electronic counter, for example, Beckmann Coulter Z2.
2. Counting pots.
3. Fixed volume dispenser (optional).
4. Ice tray.

2.6. For Cryopreservation

1. Cells requiring freezing.
2. Cell culture medium: Medium plus 10% FCS.
3. Freezing mixture: 10% DMSO/20% serum/70% medium or 10% DMSO/90% serum.
4. Trypsin-EDTA.
5. Hemocytometer or cell counter.
6. Liquid nitrogen freezer.
7. Cryotubes (freezing ampules).
8. Pipets.
9. Gloves and face mask.

3. Methods

3.1. Primary Culture

3.1.1. Initial Establishment from Solid Primary Tumor or Solid Metastasis

1. Obtain cancer material at surgery (*see* **Note 1**). Place fragments of the material into tissue culture medium, (e.g., RPMI 1640 or DMEM) in sterile plastic containers, for example, a Universal container. Keep material cold (on ice) and transfer to the tissue culture suite as rapidly as possible.

2. Within a sterile environment, for example, a Class II hood, select the most viable tissue and discard any necrotic tissue. Wash the tissue initially either with medium, PBS, or Hank's balanced salt solution (HBSS) to remove blood.
3. Two options are then available for tumor disaggregation—mechanical or enzymatic dispersion.
4. For mechanical disaggregation, cut tumor fragments into small pieces (1–2 mm diameter) by the use of crossed scalpels. This is most easily done on a Petri dish. Add a small amount of cell culture medium. Using a sterile pipet or pastette, transfer the fragments to a 25-cm^2 culture flask. If a sterilized metal sieve is available, this can be used to remove everything other than clusters of cells or single cells. To help attachment, it is often easiest to use the minimum volume of culture medium initially (2–3 mL) (*see* **Note 2**) and then add additional medium for a total volume of 5–10 mL. After several days, explants and clusters will attach to the plastic and cells will grow out from the sites of adherence.
5. Alternatively, small fragments can be broken up by the use of proteolytic enzymes. Trypsin, collagenase, hyaluronidase, elastase, dispase, and papain are all useful enzymes for this purpose. Dependent on the tissue, optimization of the enzymes and their working concentrations are required to obtain the best dissociation without excessive destruction. Sometimes, enzyme cocktails have been used overnight on ice and these may have a gentler effect (*see* **Note 3**).
6. If trypsin is used, the fragment is still chopped initially into relatively small pieces, for example, 2–3 mm diameter. The small fragments are then added to a trypsin solution (0.25%) at a density of 1 g tissue/10 mL and stirred at 37°C for 30 min. The supernatant is collected and centrifuged at 600*g* for 5 min to collect a cell pellet. This cell pellet is resuspended in full culture medium (containing serum which will inactivate the trypsin). The fragments will not necessarily disaggregate fully after a single 30-min step and the process can be repeated until most cells have become suspended in the supernatant. A modification of this process is to soak the initial fragments overnight at 4°C in the trypsin solution, allowing effective tissue penetration, and a single 30-min treatment at 37°C will be more effective. The cell suspension obtained is then centrifuged and cell culture medium (containing serum) added.
7. The other enzyme widely used for this step is collagenase as collagen is one of the major extracellular matrix proteins present in many stroma. Collagenase is added to culture medium (containing serum) at 200–1000 U/mL and can be left for 2–5 d.
8. The choice of medium to be used varies and is generally dependent on the media used in a particular laboratory. Ideally, several culture flasks should be set up using a variety of conditions as cells may grow differentially dependent on the media type. Popular media include DMEM, RPMI 1640, F12, McCoy's either alone, or in various 1/1 combinations of these. Serum at a percentage of 10% is also included. Higher concentrations of serum (up to 20%) are sometimes used and although this may be beneficial to certain cancer cell types, it may be even more beneficial to noncancer cells, such as fibroblasts, within the culture and help promote their overgrowth (*see* **Note 4**).
9. If the cells were growing within a fluid, e.g., an ascites or effusion, this can be spun down and the supernatant added at a level of 10–20% to the medium. Similarly, as the primary culture grows, the conditioned medium, i.e., medium that has been "conditioned" by the secretion of cell components, can be removed periodically and added back to fresh medium at a percentage of 10%.
10. Cultures should be monitored regularly to check which cell types adhere and grow.
11. Once there is sufficient material to maintain and expand the culture, it is essential to cryopreserve samples. Cell cultures should also be tested for the presence of mycoplasma.

3.1.2. Initial Establishment from Cell Suspensions

If the clinical tissue is a fluid such as an ascites, aspirate, or effusion the following method can be used.

1. Collect freshly obtained clinical fluids, for example, ovarian ascites, from the patient and transfer to a sterile environment. For an ovarian ascites, the volume is typically of the order of 1 L.
2. Centrifuge the fluids for 20 min at 3000*g* and 4°C to produce a cell pellet.
3. Discard the fluid and resuspend the cell pellet in PBS.
4. If the sample contains a particularly high number of red blood cells it is beneficial to remove the majority of these by centrifuging through Histopaque (or Ficoll-Paque). The tumor cell pellet is suspended in PBS or HBSS (10 mL) and placed onto Histopaque (10 mL) in a Universal container.
5. Tubes are centrifuged at 1000*g* for 20–30 min.
6. Cells at the interface of the buffer and the Histopaque are collected by pastette or pipet, resuspended in buffer, and centrifuged at 600*g* for 5 min. This wash is repeated.
7. Cells are then resuspended in cell culture medium and placed in a tissue culture flask (*see* **Notes 5**,**6**).

3.2. Routine Feeding and Maintenance

3.2.1. Adherent Monolayer

1. For monolayer cultures that are not being subcultured, remove spent media with a sterile pipet and add an equal volume of fresh media (plus serum plus any additives).
2. The periodicity of feeding depends on the growth rate of the primary culture or cell line. In general, feeding 2–3 times/wk is recommended.

3.2.2. Suspension Culture

1. For suspension cultures, medium containing cells must be centrifuged in a sterile manner, at 600*g* for 5 min in a Universal container.
2. The cell pellet is resuspended in fresh medium.
3. Often, if media is being depleted as a result of growth, then the culture can simply be divided 1/5–1/10 into fresh complete-culture medium without the necessity to spin cells down.

3.3. Subculture of Cells

3.3.1. Monolayer Cells

Adherent cells that are in late log phase require subculturing to maintain optimal growth (*see* **Note 7**).

1. Check the culture to ensure cells are in late log/early plateau phase (80–90% of the surface area is covered) and confirm that the cells are healthy and free of contamination.
2. Remove the cell culture medium by pipet and discard.
3. Wash cells twice with PBS to remove traces of serum that will inactivate trypsin and discard.
4. Add 1–2 mL trypsin-EDTA to a 25-mL flask (and scale up accordingly for larger sized flasks). Swirl the solution across the monolayer to ensure the trypsin reaches all cells. Return the flask to the incubator for 5–10 min (*see* **Note 8**).
5. Check the detachment of the cells at intervals. This is best done using a microscope, but with experience the monolayer sheet can be seen to disperse macroscopically. The cells

should not be left in trypsin for long periods once detached. Fresh culture medium containing serum is then added to inactivate the trypsin in the cell suspension. Pipeting or syringing this suspension will then help break up any cell clusters into single cells.
6. The cell suspension can then either be counted if an accurate cell density is required at subculture, or the suspension can then be "split" into an appropriate ratio. For example, it is often convenient to split the cells 1/5 or 1/10 depending on growth rates.
7. For a 1/10 ratio, a 1/10 aliquot of the cell suspension would be placed into a new flask with the full amount of cell culture medium required for that flask size. Subculturing from primary cultures should involve relative small split ratios, e.g., 1/2 or 1/3. Cell culture flasks are then returned to the CO_2 incubator. After 24 h, the culture should be checked to ensure that cells are reattaching and the pH of the medium is approx pH 7.4 (*see* **Note 9**).
8. Medium is then changed as necessary until the next subculture.

3.3.2. Suspension Culture

For cells growing in suspension, subculturing does not require the trypsinization steps needed for harvesting adherent cells.

1. The cell suspension is first checked to ensure cells are in late log/early plateau phase and to confirm that the cells are healthy and free of contamination (*see* **Note 10**).
2. The cell suspension can then either be counted if an accurate cell density is required at subculture, or the suspension can then be "split" into an appropriate ratio. For example, it is often convenient to split the cells 1/5 or 1/10 depending on growth rates. For a 1/10 ratio, a 1/10 aliquot of the cell suspension would be placed into a new flask with the full amount of cell culture medium required for that flask size.
3. Cell culture flasks are then returned to the CO_2 incubator. After 24 h, the culture should be checked to ensure that the pH of the medium is approx pH 7.4 (*see* **Note 9**).

3.4. Cloning

3.4.1. Dilution Cloning on Plastic

For monolayer cultures, trypsinize and harvest cells to prepare a cell suspension as described in **Subheading 3.3.1.** This step is unnecessary for cells growing in suspension. Pass cells through a pipet, pastette, or needle to produce a single cell suspension.

1. Dilute cells to a range of low concentrations, e.g., 100, 30, and 10 cells/mL.
2. Place cell suspensions into individual wells of a 24–well plate (1 mL/well).
3. Allow colonies to grow. Typically this will take 3–4 wk. Change media as growth commences.
4. Inspect each well to identify wells in which a single colony has grown. It may be necessary to repeat the range of cell concentrations plated to encompass either a higher or lower range.
5. Wells in which a single colony is present are then expanded by subculture as described above (*see* **Subheading 3.3.**). They should be designated carefully to indicate a link with the parental culture.

3.4.2. Cloning in Agar

Cancer cells can conveniently be cloned in agar as they demonstrate anchorage independent growth. This provides one the means of allowing the malignant cells to

grow in the presence of nontransformed cells that will generally not grow under these conditions.

1. Prepare agar. Agar solutions can be prepared prior to use. A 5% solution of agar in distilled water is dispensed into glass Universal containers. Autoclave for 15 min. The agar solution should be kept at 45°C or higher to prevent it from forming a gel.
2. For monolayer cultures, trypsinize and harvest cells to prepare a cell suspension as described in **Subheading 3.3.1.** This step is unnecessary for cells growing in suspension. Passage cells through a pipet, pastette, or needle to produce a single cell suspension. Count cells. Prepare a cell suspension at 2.5X final density.
3. Cell culture media (for example, Ham's F12 plus 10% FCS) is warmed to 37°C.
4. Using a pipet or pastette, add 2 mL of 5% agar to 18 mL cell culture medium in a prewarmed glass-Universal and mix thoroughly.
5. Add 3.6 mL of this 0.5% agar solution to 2.4 mL of the cell suspension in individual tubes (with caps that allow diffusion) and mix thoroughly.
6. Place tubes on ice for 5 min and close caps. Then place tubes into a CO_2 incubator at 37°C.
7. After 1 wk, 1 mL fresh medium plus serum can be added to each tube. The tube is re-fed weekly until colony size is greater than 50 cells.
8. Periodically, tubes are inspected to determine whether colonies have formed. A tube is selected, liquid medium removed from the tube, and the agar plug is placed into an empty Petri dish. The plug is pressed down to allow viewing through an inverted microscope.

3.4.3. Estimation of Colony Forming Efficiency (CFE) on Plastic

1. Monolayer cells are trypsinized as described in **Subheading 3.3.1.** and a cell count is taken of the cell suspension (*see* **Subheading 3.5.**).
2. Cell dilutions are prepared with differing numbers of cells (e.g., 10,000, 3000, 1000, 300, and 100 cells/2 mL).
3. Dispense 2 mL aliquots into Petri dishes or individual wells of a 6-well plate.
4. Allow cells to attach.
5. Replace full culture medium 2–3 times/wk.
6. When sufficient time has elapsed to allow colonies (>50 cells) to form (approx several weeks) and dependent on the growth rate of the cell line, colonies are counted.
7. It is generally convenient to count wells containing approx 100–200 colonies. At higher densities, colonies will start to merge and it will be unclear as to whether colonies developed from single cells or have merged. Colonies can also be stained with dyes such as hematoxylin or crystal violet to aid counting.

3.5. Cell Counting

3.5.1. Hemocytometer Counting

1. Trypsinize monolayer cells until they are detached from the plastic (*see* **Subheading 3.3.**) and then add medium containing FCS to inhibit cell damage by over-trypsinization. This step is not necessary for suspension cultures.
2. Ensure that the cells are in single-cell suspension. This should be done by repeatedly drawing the cells into a pastette or a syringe and checking the appearance of a drop of cells under the microscope (*see* **Note 11**).
3. Prepare the hemocytometer and cover slip. These should be clean and wiped with 70% alcohol (*see* **Note 12**). Take care not to scratch the silvered surface.
4. Slightly moisten the hemocytometer and cover slip (*see* **Note 13**) and place the cover slip over the grid. Gently move the cover slip back and forth resulting in its attachment to the

hemocytometer and the appearance of Newton's rings (rainbow colors like those formed by oil on water).

5. The hemocytometer is now ready to be filled. Place a pipet filled with a well-suspended mix of cells at the edge of the coverslip and then by slowly expelling some contents, draw the fluid into the chamber by capillary action.
6. Obtain the cell concentration by counting cells in the grid area. Several choices are available depending on the density of the cells:
   a. Count the 25 squares within the large middle square. Total cell count in 25 squares × 10,000 = number of cells/mL.
   b. Count the number of cells in the 4 outer squares. Total cell count in 4 squares × 2500 = number of cells/mL. The choice of methods is dependent on the cell concentration. The accuracy of the procedure depends upon the number of cells counted.
7. Each hemocytometer normally has two grid areas and it is good practice to count both and use the mean count to calculate cell number.
8. One advantage of using the hemocytometer method is that it allows for a variation of technique involving the use of Trypan blue dye to enable differentiation between dead/damaged cells and the healthy viable cell population.

3.5.2. Viable Cell Counting Using Trypan Blue

Trypan blue is a dye that does not interact with the cell unless the cell membrane is damaged. Healthy undamaged cells exclude the dye, but it is readily absorbed by damaged cells and renders them clearly visible (blue) under the microscope.

1. A 0.5-mL aliquot of cell suspension, obtained as previously described, is incubated with 0.5 mL of 0.4% Trypan blue dye (5 min at room temperature).
2. Cells are counted using the normal hemocytometer protocol and the percentage of dead or damaged cells can be established.

3.5.3. Electronic Counting

Detailed operating manuals and training are provided by Beckman Coulter. The following is a brief operating summary:

1. Switch on instrument 30 min prior to use. To avoid build up of debris in the pipe-work the instrument is routinely emptied after each use (daily) and refilled with Coulter Clenz. This is removed and the empty instrument refilled with saline. This is done automatically by making selections from the menu. Prepare counting pots by adding the required volume of PBS (typically 9.8 mL, allowing samples to be added in 0.2 mL increments). The use of an automatic dispensor makes this task much easier.
2. Instrument parameters are set on the set up page. These include sample volume particle size range (upper and lower thresholds) and the number of counts per sample.
3. Cells should be harvested and trypsin inactivated by adding an equal volume of FCS-containing media. Where necessary cells are agitated to give a single cell suspension (*see* **Note 11**). Cell samples contained in 24 well trays may be stabilized for longer by placing them on an ice tray.
4. Measure 0.2 mL of cell suspension into each counting pot just prior to counting. Mix gently by rolling or inverting. Do not shake vigorously as this will create air bubbles which may be counted by the instrument.

5. Flush aperture by selecting this option from the start-up menu. Check background by counting a blank sample. If this is unacceptably high repeat the flushing step.
6. Count the sample. Repeat process for further samples. It is not necessary to flush the aperture between replicate samples.
7. The results may be directed to a printer or to a computer.

3.6. Cryopreservation

3.6.1. Freezing

1. Cells that are typically in log phase of growth should be used, and ideally medium should be replaced 24 h prior to freezing.
2. For monolayers, cells should first be washed with PBS (after removal of culture medium) and then treated with trypsin-EDTA (5 mL/75 cm^2 flask) to detach the monolayer.
3. After confirmation of detachment by observation through a microscope, trypsin should be inactivated by addition of culture medium (containing 10% serum).
4. An aliquot of the cell suspension is then removed and a cell count taken using either a hemocytometer or cell counter.
5. Cells are then pelleted by centrifugation (5 min at 600*g*) and resuspended in freezing mix (kept cold on ice)(*see* **Note 14**) at a density of approx 10^7 cells/mL (*see* **Note 15**).
6. The cell suspension is then aliquoted into freezing ampules, e.g., cryotubes or other appropriate freezing vials (*see* **Note 16**). These vials should be labeled with the essential information, including at least the cell name, passage number, date, and the name of the individual storing the vials
7. Ampules are then placed into the freezing chamber of a programmable freezer if this is available. A simple alternative is to place ampules into a polystyrene container and then put this into a –70°C/-80°C freezer overnight. This achieves a cooling rate that approximates 1°C/min. Once ampules are at –70°C to –80°C, they can be transferred directly to liquid nitrogen (*see* **Note 17**).

3.6.2. Recovery and Thawing

1. When thawing, cells should be warmed rapidly by removing an ampule from the liquid nitrogen and placing it into a 37°C water bath (*see* **Note 18**). Ampules should be washed with 70% ethanol before opening.
2. Once thawed the freezing solution should be placed into 10% serum/90% medium (1 mL freezing solution into 20 mL serum/medium) and spun down at 600*g* for 5 min. This may be repeated and this will effectively remove the DMSO from the cells (*see* **Note 19**).

4. Notes

1. If sufficient material is available, it is desirable to store some of the primary material in liquid nitrogen. This is useful for later characterization.
2. The addition of antibiotics in media added to primary cultures is also a useful precaution and combinations such as penicillin/streptomycin are widely used.
3. Various cocktails of digestive enzymes are used. For example, the cocktail suggested in Chapter 8 for brain tissues include 0.002% DNase type I, 0.01% hyaluronidase type V, and 0.1% collagenase type IV in RPMI 1640 and tissue is treated at 37°C for 1–3 h.
4. A number of strategies are used to help isolate and selectively aid the growth of cancer cells compared to stromal (especially fibroblastic) cells when initially in culture. If the

fibroblasts rapidly adhere but clusters of cancer cells are more loosely attached, these clusters can be made to detach by tapping the flask. The floating clusters are then collected by centrifugation of the medium and resuspended in a new flask. Similarly, trypsinization can be used to separate rapidly attaching/detaching cells from more adherent cell types. Finally, if clear cancer colonies are viewed by microscope in a "sea" of fibroblasts, then after marking the positions of colonies with a marker pen on the base of the flask, use a scraper to mechanically remove the fibroblast overgrowth.

5. Cells from suspensions can be cryopreserved at the outset to allow culture to be set up at a later time.
6. The use of a higher percentage of serum (15–20%) can be helpful at the outset. Depending on the source of the culture, "conditioned medium " that the cells were growing in, e.g., ascitic or pleural effusion fluid, may provide some benefit and can be added at a level of 10% final volume.
7. Subculturing is best performed when cells are still in log phase and at their healthiest.
8. The cells should be trypsinized for the minimum period necessary. This will vary from cell-line to cell-line and may vary from 1 min to longer than 20 min. Tapping the flask when cells have rounded up will also help detachment.
9. Different cell lines will take varying amounts of time to reattach after trypsinization. Many cell lines will have reattached almost completely within 24 h but others take longer. As the cell density is relatively low after subculturing it is important to ensure that the media is well gassed; otherwise, it will become alkaline as the pH increases.
10. When growing in suspension live cells are typically bright when viewed under phase contrast. Viability can be assessed by use of a vital dye such as Trypan blue (*see* **Subheading 3.5.2.**).
11. Most cell lines will readily form single-cell suspension by repeated agitation with a pastette. If this is not sufficient then a 10-mL syringe with an attached needle should be used. Begin with a wide-bore needle and then try smaller-bore needles until a satisfactory suspension is achieved. If a number of samples are to be counted, for example, from a 24-well tray, then it is best to prepare only a few samples at a time as some cells may reclump if left sitting for a prolonged period.
12. If the hemocytometer technique is routinely used, the counter and cover slips may be stored in a small volume of 70% ethanol after cleaning, in readiness for next use.
13. When moistening the counter and cover slip do not overwet them. It is usually sufficient to breathe on the counter or the cover slip prior to bringing them into contact. If this does not work, then cooling the counter by running cold water over it may help. Staining of cells often facilitates visualization and counting. Cells should be treated with 10% formalin (time/conditions) and then stained with Trypan blue or other stain to increase visibility of the cells.
14. The freezing mixture can vary in composition. The key determinant is for the DMSO to be present at 10%. Serum percentages can then vary from 20% to 90% with medium making up the difference. Glycerol can be used instead of DMSO.
15. Cells are frozen at high cell densities and also recovered at high density.
16. Either plastic or glass ampules can be used. Plastic ampules have the advantage of being presterilized and are easy to label; glass ampules, when sealed, are more likely to exclude nitrogen.
17. Cells can be preserved in liquid nitrogen either in the liquid phase (–196°C) or in the vapor phase (–120°C to –156°C).

18. Care should be taken on thawing, as liquid nitrogen may cause ampules to explode. Liquid nitrogen is hazardous and gloves and protective face equipment should be used when handling it. DMSO can penetrate skin and carry dissolved products across the skin barrier, so it should be handled with caution.
19. When a batch of cells is stored away, it is good practice to thaw one of the ampules to confirm viability.
20. General safety considerations include the following:
    a. All cell culture work should be undertaken in a microbiological safety cabinet, preferably Class II.
    b. Aseptic technique should be used at all times in this cabinet.
    c. The work surfaces should be sprayed with alcohol (or similar decontaminant) before and after use.
    d. All biological waste should be autoclaved and liquids or media treated with bleach.
    e. Mouth pipeting should be avoided.

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