ANIMAL

TISSUE CULTURE LAB

Prepared by

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CONTENT

LABORATORY SAFETY GENERAL

RULES AND REGULATIONS

Introduction:

A rewarding laboratory experience demands strict adherence to prescribed rules for personal and environmental safety. The former reflects concern for your personal safety in terms of avoiding laboratory setting to prevent contamination of experimental procedures by microorganisms from external sources. Because most microbiological laboratory procedures require the use of living organisms, an integral part of all laboratory session is the use of aseptic techniques. Although the virulence of microorganisms used in the academic laboratory environment has been greatly diminished because of their long-term maintenance on artificial media, all microorganisms should be treated as potential pathogens (organisms capable of producing disease). Thus, microbiology students must develop aseptic techniques (free of pathogenic organisms) in preparation for industrial and clinical marketplaces where manipulation of infectious organisms may be the norm rather than the exception.

The following basic steps should be observed at all times to reduce the ever-present microbial flora of the laboratory environment.

1. Upon entering the laboratory, place coast, books, and other paraphernalia in specified locations-never on bench tops.

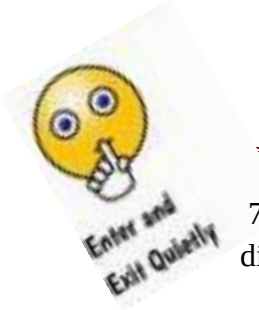
2. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.

3. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant solution provided by the instructor.

4. Do not place contaminated instruments, such as inoculating loops, needles, and pipettes, on bench tops. Loops and needles should be sterilized by incineration, and pipettes should be disposed of in designated receptacles.

5. On completion of the laboratory session, place all cultures and materials in the disposal area as designated by the instructor.

6. Rapid and efficient manipulation of fungal cultures and materials in the disposal area as designated by the instructor.



7. Rapid and efficient manipulation of fungal cultures is required to prevent the dissemination of their reproductive spores in the laboratory environment.

To prevent accidental injury and infection of yourself and others, observe following regulations at all times:

1. Wash your hands with liquid detergent and dry them with paper towels upon entering and prior to leaving the laboratory.

2. Wear a paper cap or tie back long hair to minimize its exposure to open flames.

3. Wear a lab coat while working in the laboratory to protect clothing from contamination or accidental discoloration by staining solutions.

4. Never apply cosmetics or insert contact lenses in the laboratory.

5. Do not smoke, eat, or drink in the laboratory. These activities are absolutely prohibited.

6. Carry cultures in a test - tube rack when moving around the laboratory. Likewise, keep cultures in a test-tube rack on the bench tops when not in use. This serves a dual purpose to prevent accidents and to avoid contamination of yourself and the environment.

7. Never remove media, equipment, or especially, bacterial cultures from the laboratory. Doing so is absolutely prohibited.

8. Immediately cover spilled cultures or broken cultures tubes with paper towels and then saturate them with disinfectant solution. After 15 minutes of reaction time, remove the towels and dispose of them in a manner indicated by the instructor.

9. Report accidental cuts or burns to the instructor immediately.

10. Never pipette by mouth any broth cultures or chemical reagents. Doing so is strictly prohibited. Pipetting is to be carried out with the aid of a mechanical pipetting device.

11. Do not lick labels. Use only self-stick labels for the identification of experimental cultures.

12. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.

I have read the above laboratory safety rules and agree to abide them.

INTRODUCTION TO ANIMAL CELL CULTURE LABORATORY

Cell culture is an indispensable technique for understanding the structure and function of cells, in recent times it has very good implications in biotechnology. Cultured animal cells are commercially used for the production of interferon, vaccines and clinical materials like growth hormones and urokinase. In the process of learning the techniques of cell culture and gene transfer you will become familiar with several terminologies and hypotheses.

Make yourself familiar with the equipments (incubators, centrifuges and microscope etc.,).

If you have problems, approach the faculty members.

Good laboratory work habits will help you a grand success. Follow the following guide lines very strictly. These will protect you and your experiments.

1. No eating and drinking in the lab.

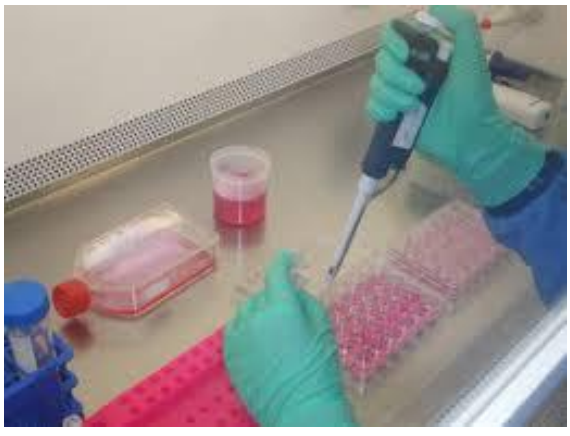
2. No storage of food materials in the lab.

3. No mouth pipetting. Use appropriate pipetting aids that are available.

4. Wear gloves for certain experiments whenever it is necessary.

5. Work cleanly in an organized manner. Wipe your tissue culture hood bench with 70% ethanol.

6. Most important, label everything you use with your Reg. number, date and name of the reagent, buffer or medium.



STERILIZATION TECHNIQUES

AIM : To prepare the materials required for various cell culture practices in sterile condition.

INTRODUCTION :

The term control as used here refers to the reduction in numbers and or activity of the total microbial flora. The principal reasons for controlling microorganisms and to prevent transmission of disease and infection, to prevent contamination and to prevent deterioration and spoilage of materials by microorganisms.

Microorganisms can be removed , inhibited or killed by various physical agents, physical processes or chemical agents. A variety of techniques and agents are available,and they act in many different ways.

Steam under pressure: Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization. Steam under pressure provides temperatures above those obtainable by boiling as shown in Table 22-5. In addition, it has the advantages of rapid heating, penetration, and moisture in abundance, which facilitates the coagulation of proteins.

Preparation and Sterilization of Media:

During the preparation of complex solutions, care must be taken to ensure that all of the constituents dissolve and do not get filtered out during sterilization and that they remain in solution after autoclaving or storage. Concentrated media are often prepared at a low pH (between 3.5 and 5.0) to keep all the constituents in solution, but even then some precipitation may occur. If the constituents are properly re-suspended, they will usually redissolve on dilution, but if the precipitate has been formed by degradation of some of the constituents of the medium, then the quality of the medium may be reduced. If a precipitate forms, the performance of the medium should be checked by cell growth and cloning and an appropriate assay of special functions or the medium should be replaced.

Commercial media are supplied as :

(1) working-strength solutions (1×) with or without glutamine;

(2) 10× concentrates, usually without $NaHCO_3$ and glutamine, which are available as separate concentrates;

(3) powdered media, with or without $NaHCO_3$ and glutamine.

Powdered media are the cheapest and not a great deal more expensive than making up medium from your own chemical constituents if you include time for preparation, sterilization, and quality control and for the cost of raw materials of high purity and the cost of overheads such as power and wages. Powdered media are quality controlled by the manufacturer for their growth-promoting properties but not, of course, for sterility. They are mixed very efficiently by ball milling, so, in theory, a pack may be subdivided for use at different times. However, in practice, it is better to match the size of the pack to the volume that you intend to prepare, because once the pack is opened, the contents may deteriorate and some of the constituents may settle.

TABLE 10.1. Methods of Sterilization

Method	Conditions	Materials	Limitations
Dry heat	160°C, 1–2 h	Heat stable: metals, glass, PTFE	Some charring may occur, e.g., of indicating tape and cotton plugs
Steam	121°C, 15–20 min	Heat-stable liquids: water, salt solutions, autoclavable media; moderately heat-stable plastics: silicones, polycarbonate, nylon, polypropylene	Steam penetration requires steam-permeable packaging; large fluid loads need time to heat up
Irradiation			
γ-Irradiation	25 kGy	Plastics, organic scaffolds, heat-sensitive reagents and pharmaceuticals	Chemical alteration of plastics can occur; macromolecular degradation
Electron beam	25 kGy	Plastics, organic scaffolds, heat-sensitive reagents and pharmaceuticals	Needs high-energy source; not suitable for average laboratory installation
Microwave	5 min full power	Aqueous solutions and gels such as agar	Only useful for small volumes; usually just for melting agar
Short-wave UV	254 nM, 50–100 W, 30 min	Flat surfaces, circulating air	Will not reach shadow areas. Spores resistant
Chemical			
Ethylene oxide	1 h	Heat-labile plastics	Items must be ventilated for 24–48 h; leaves toxic residue
Hypochlorite	300–2500 ppm 30 min	Contaminated solutions; plastics	Needs extensive washing. May leave residue
70% Alcohol	Soak for 1 h	Dissecting instruments (combined with flaming); some plastics	Does not kill spores; fire risk with flaming; precast Perspex or Lucite may shatter if immersed in alcohol
Filtration	0.1- to 0.2-μm porosity	All aqueous solutions; particularly suitable for heat-labile reagents and media; specify low protein binding for growth factors, etc	Not suitable for some solvents, e.g., DMSO; slow with viscous solutions

Cell Culture Media preparation

Introduction:

Cell culture media are complex mixtures of salts, carbohydrates, vitamins, amino acids, metabolic precursors, growth factors, hormones, and trace elements.

The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Carbohydrates are supplied primarily in the form of glucose. In some instances, glucose is replaced with galactose to decrease lactic acid build-up, as galactose is metabolized at a slower rate. Other carbon sources include amino acids (particularly L-glutamine) and pyruvate. In addition to nutrients, the medium helps maintain the pH and osmolality in a culture system. The pH is maintained by one or more buffering systems; CO_2/sodium bicarbonate, phosphate, and HEPES are the most common. Serum will also buffer a complete medium. Phenol red, a pH indicator, is added to medium to colorimetrically monitor changes in pH. Commonly used culture media include the following:

1) **Eagle's Minimum Essential Medium (EMEM)** was among the first widely used media and was formulated by Harry Eagle from his earlier and simpler basal medium (BME). BME was developed for culturing Hela cells. Over time, there have been numerous variations on the EMEM formula for different applications. EMEM contains Earle's balanced salt solution, nonessential amino acids, and sodium pyruvate. It is formulated with a reduced sodium bicarbonate concentration (1,500 mg/l) for use with 5% CO_2. Because EMEM is a simple medium, it is often fortified with additional supplements or higher levels of serum.
2) **Dulbecco's Modified Eagle's Medium (DMEM)** has roughly twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids (though not all nonessential amino acids). The original formulation contained 1,000 mg/L of glucose, but in the more commonly used variations this amount was increased to 4,500 mg/L. DMEM (ATCC® No. 30-2002) has 4,500 mg/L of glucose and a reduced sodium bicarbonate concentration (1,500 mg/L) for use with 5% CO_2.
3) **DMEM/F12 Medium** is a 1:1 mixture of Dulbecco's modified EMEM and Ham's F-12. It is an extremely rich and complex medium and will support the growth of a broad range of cell types in both serum and serum free formulations. DMEM/F12 medium has a reduced sodium bicarbonate concentration (1,500 mg/L) for use with 5% CO_2.
4) **RPMI-1640** was developed at Roswell Park Memorial Institute (RPMI) in Buffalo, New York.. RPMI-1640 will support the growth of a wide variety of

cells in suspension as well as a number of cells grown as monolayers. Complete Growth Media RPMI-1640 was modified to contain higher amounts of glucose (4,500 mg/L), sodium pyruvate, and HEPES buffer. It also contains a reduced concentration of sodium bicarbonate (1,500 mg/L) for use with 5% CO_2.

Dulbecco' Modified Eagle's Media (DMEM).

1) Prepare 900 ml of distilled water in clean graduated cylinder. Water temperature should be 15-30 C.
2) Put the beaker on magnetic stirrer.
3) Add 15.4g/l DMEM media powder to the water and stir gently. Stir till completely dissolved.
4) Add 1.2 g Sodium Bicarbonate (or 49.1 ml of 7.5 % Sodium Bicarbonate solution).
5) Add 10% (100 ml) of fetal bovine serum.
6) Place FBS in water bath so it doesn't prevent cell growth.
7) Add 10ml from antibiotics (penicillin, streptomycin).
8) Adjust PH to 0.2-0.3 units below the required PH using 1N HCL or 1N NAOH. The PH will rise by 0.1-0.3 units after filtration.
9) The required media pH is 7.4.
10) Add distilled water up to 1 Liter.
11) Filter for sterility with 0.2micron filter into sterile bottles

RPMI 1640 culture media preparation:

1) Prepare 900 ml of distilled water in clean graduated cylinder. Water temperature should be 15-30 C.
2) Put the beaker on magnetic stirrer.
3) Add equivalent powder to the water and stir gently. Stir till completely dissolved.
4) Add 20 g Sodium Bicarbonate (or 26.67ml of 7.5 % Sodium Bicarbonate solution).
5) Adjust PH to 0.2-0.3 units below the required PH using 1N HCL or 1N NAOH. The pH will rise by 0.1-0.3 units after filtration.
6) Add distilled water up to 1 Liter.
7) Filter for sterility with 0.2micron filter into sterile bottles.

Trypsin EDTA (TRED)

An enzyme used to detach the cells from a culture dish. EDTA binds Calcium ions in the media that would normally inhibit trypsin.

Phosphate Buffered Saline

Used to wash/ remove excess serum that inhibits the function of TRED. Must be warmed in the water bath before use so cells are not shocked by cold liquid.

Routine cell culture maintenance

Introduction:

Once a culture is initiated, whether it is a primary culture or a subculture of a cell line, it will need a periodic medium change(feeding or refreshing) followed eventually by subculture if the cells are proliferating. In non-proliferating cultures, the medium will still need to be changed periodically, as the cells will still metabolize and some constituents of the medium will become exhausted or will degrade spontaneously. Intervals between medium hanges and between subcultures vary from one cell line to another, depending on the rate of growth and metabolism. Rapidly growing transformed cell lines, such as HeLa, are usually subcultured once per week, and the medium should be changed four days later. More slowly growing, particularly non-transformed, cell lines may need to be subcultured only every two, three, or even four weeks, and the medium should be changed weekly between subcultures.

Significance of Cell Morphology:

The cells should also be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate. Such signs may imply that the culture requires a medium change, or may indicate a more serious problem, such as inadequate or toxic medium or serum, microbial contamination, or senescence of the cell line.

Four factors indicate the need for the replacement of culture medium:

1. **A drop in pH.**
   Most cells stop growing as the pH falls from pH 7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed.

2. **Cell concentration**. Cultures at a high cell concentration exhaust the medium faster than those at a low concentration. This factor is usually evident in the rate of change of pH, but not always.

3. **Cell type**. Normal cells (e.g., diploid fibroblasts) usually stop dividing at a high cell density ,because of cell crowding, shape change, growth factor depletion, and other reasons.

4. **Morphological deterioration.** This factor must be anticipated by regular examination and familiarity with the cell line. If deterioration is allowed to progress too far, it will be irreversible, as the cells will tend to enter apoptosis.

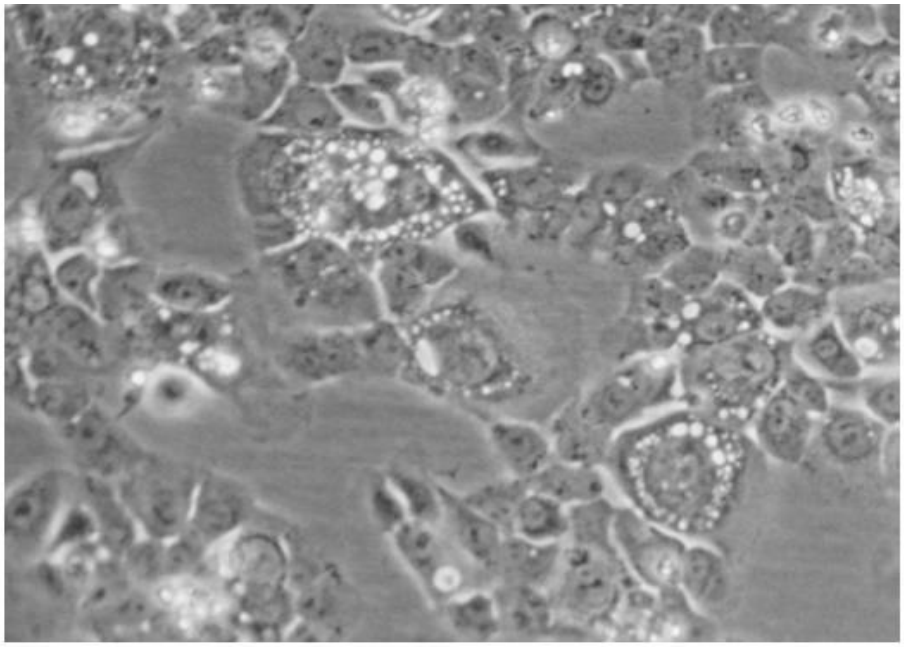


Fig. 12.1. Unhealthy Cells. Vacuolation and granulation in bronchial epithelial cells (BEAS-2B) due, in this case, to medium inadequacy. The cytoplasm of the cells becomes granular, particularly around the nucleus, and vacuolation occurs. The cells may become more refractile at the edge if cell spreading is impaired.

Volume, depth, and surface area.

The usual ratio of medium volume to surface area is 0.2 to 0.5 mL/cm2 . The upper limit is set by gaseous diffusion through the liquid layer, and the optimum ratio depends on the oxygen requirement of the cells. Cells with a high O2 requirement do better in shallow medium (e.g., 2 mm), and those with a low requirement may do better in deep medium (e.g.,5mm).If the depth of the medium is greater than 5mm, then gaseous diffusion may be come limiting. With monolayer cultures, this problem can be overcome by rocking or rolling the bottle or by perfusing the culture with medium and arranging for gas exchange in an intermediate reservoir.

Feeding a monolayer culture

Materials:

1. DMEM MEDIA
2. Sterile pipette
3. Alcohol 70%

4. Hood
5. Cell culture

Protocol:

1) Place the medium bottle in water bath at 37 C.
2) Prepare the hood by washing and clear with 70% alcohol.
3) Examine the culture carefully for signs of contamination or deterioration.
4) Check the culture by inverted microscope to detection of cell concentration and growing.
5) Take the culture to the sterile work area.
6) Uncap the flask.
7) Remove the media by pipetting.
8) Change the pipette.
9) Add the same volume of fresh medium as was removed.
10) Return the culture to the incubator.

Subculture and cell lines

Aim :

To develop secondary growth or established cells from primary culture by repeated subculture.

Introduction:

The need to subculture a monolayer is determined by the following criteria:

(1) Density of culture. Normal cells should be subcultured as soon as they reach confluence. If left more than 24 h beyond this point, they will withdraw from the cycle and take longer to recover when reseeded. Transformed cells should also be subcultured on reaching confluence or shortly after; although they will continue to proliferate beyond confluence, they will start to deteriorate after about two doublings, and efficiency will decline. Some epithelial cell lines, such as Caco-2, need to be subcultured before they reach confluence as they become too difficult to trypsinize after confluence.

(2) Exhaustion of medium. Exhaustion of the medium (see Section12.3.2)usually indicates that the medium requires replacement but if a fall in pH occurs so rapidly that the medium must be changed more frequently, then subculture may be required. Usually a drop in pH is accompanied by an increase in cell density, which is the prime indicator of the need to subculture.

(3) Time since last subculture. Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved and monitored. If cells have not reached a high enough density (i.e., they are not confluent) by the appropriate time, then increase the seeding density, or if they reach confluence too soon, then reduce the seeding density. Determination of the correct seeding density and subculture interval is best done by performing a growth curve Once this routine is established, the recurrent growth should be consistent in duration and cell yield from a given seeding density. Deviations from this pattern then signify a departure from normal conditions or indicate deterioration of the cells. Ideally, a cell concentration should be found that allows for the cells to be subcultured after 7 days, with the medium being changed after 4 days.

(4) Requirements for other procedures. When cells are required for purposes other than routine propagation, they also have to be subcultured in order to increase the stock or to change the type of culture vessel or medium. Ideally this procedure is done at the regular subculture time, when it is known that the

culture is performing routinely, what the reseeding conditions are, and what outcome can be expected. However, demands for cells do not always fit the established routine for maintenance and compromises have to be made. but, 1) cells should not be subcultured while still within the lag period, and2)cells should always be taken between the middle of the log phase and the time at which they will enter the plateau phase of a previous subculture.

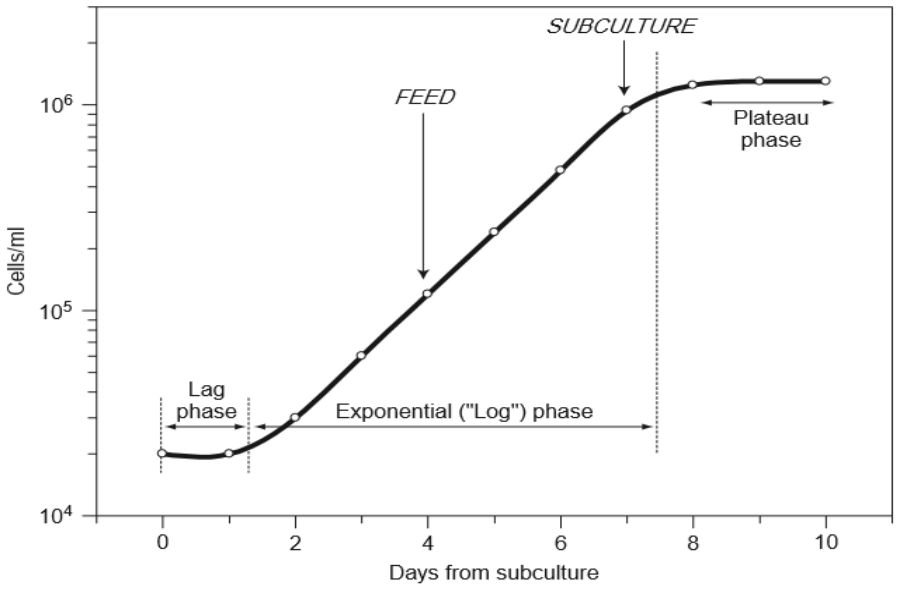


Fig. 12.2. Growth Curve and Maintenance. Semilog plot of cell concentration versus time from subculture, showing the lag phase, exponential phase, and plateau, and indicating times at which subculture and feeding should be performed (*see also* Section 20.9.2; Fig. 20.8).

Materials:

Sterile:

- Cell culture
- Culture flasks T25 ,T75 or T150
- Growth medium
- D-PBS without Calcium and magnesium
- Trypsin 0.25% with 2.21 Mm EDTA in HBBS
- Pipettes ,2ml ,5 ml, 25 ml

Non-sterile:

- Gloves
- Pipette aid
- Alcohol 70% in spray bottle.

Protocol:

1) Prepare the hood by swabbing with 70% alcohol.
2) Collect materils and reagents and replace them in biosafety hood, swab with 70% alcohol.
3) Defrost trypsin to 4C in fridge, and prewarm the medium and D-PBS in water bath at 37C.
4) Retrieve culture flask from the incubator.
5) Examine cells on an inverted microscope, look for signs of cell deterioration or contamination.
6) Check criteria for subculture: ~70% confluency and otherwise healthy looking cells.
7) Place cuture flask in biosafety hood.
8) Remove the medium in the flask
9) Add 2 ml trypsin/EDTA in the culture flask and then remove it.
10) Add 3 ml trypsin/EDTA in the culture flask and add 6 ml from DMEM media.
11) Placethe culture flask in the incubator for 3 mins.
12) Return the culture flask into the hood.
13) Transfer what's contained in the culture flask into a centrifuge tube.
14) Place the tube in the centrifuge for 10min at 1110 rpm.
15) Remove the supernatant.
16) Add 6 ml from DMEM media to tube.
17) Transfer 3 ml from the cells and media to a culture flask.
18) Complete the volume of DMEM into 10 ml in each culture flask.
19) Return to incubator.

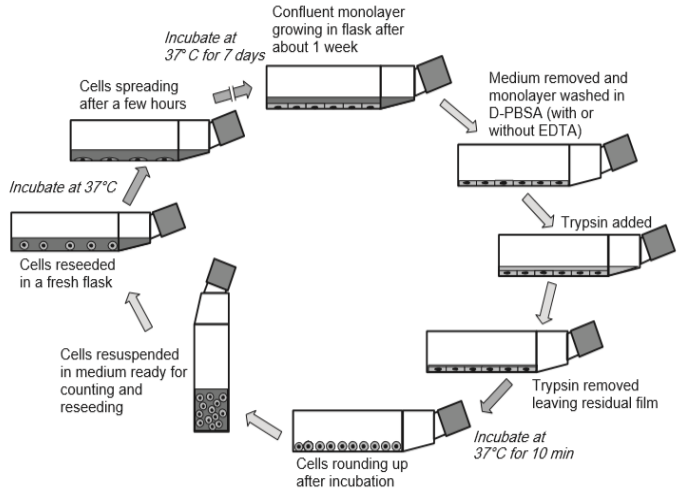


Fig. 12.3. Subculture of Monolayer. Stages in the subculture and growth cycle of monolayer cells after trypsinization (*see also* Plates 4, 5).

Cell Proliferation Assay (MTT assay)

Introduction

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells.

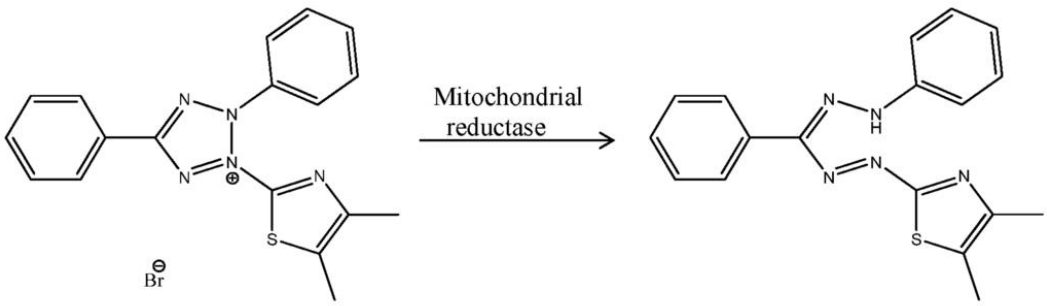
Objectives:

1- To measure the metabolic activity of cells.

2- To study the anti-proliferative effects of chemical and biological compounds on cancer cells **.**

Principle:

The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.



Tetrazolium salt
(yellow colour)

Formazan crystal
(purple colour)

Materials' and equipment's:

1-Cells to be test.
2- MTT solution
3-Solubilizing solution
4-Sterial tips
5-Sutibal media
6-96 well plate
7-Compound to be test
8-A microtiter plate reader (ELISA reader).

Experimental Protocol:

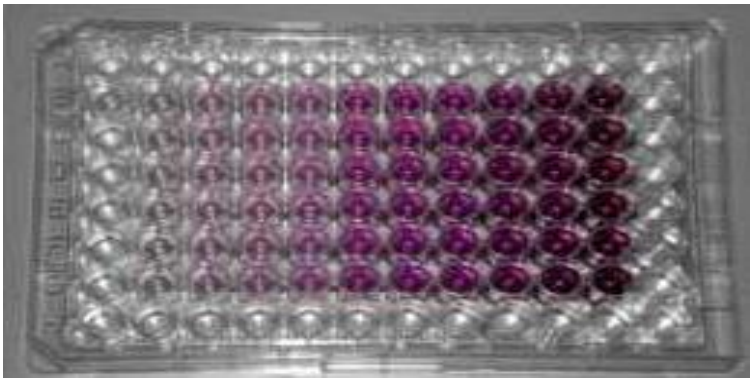
Reagent Preparation:

1-Prepare a 12 mM MTT stock solution by adding 1 mL of sterile PBS to one 5 mg vial of MTT. Mix by vortexing or sonication until dissolved. Each 5 mg vial of MTT provides sufficient reagent for 100 tests, using 10 µL of the stock solution per well. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light.

2-Add 10 mL of 0.01 M HCl to one tube containing 1 gm of SDS. Mix the solution gently by inversion or sonication until the SDS dissolves. Once prepared, the solution should be used promptly. Each tube makes sufficient solution for 100 tests, using 100 µL per well.

MTT Assay Protocol:

1-Prepare cells and test compounds in 96-well plates containing a final volume of 100 µl/well.
2-Incubate for desired period of exposure.
3-Add 10 µl MTT Solution per well and incubate it 4 hours at 37°C.
4-Add 100 µl Solubilization solution to each well to dissolve formazan crystals, mix to ensure complete solubilization and incubate it overnight .
5-Record absorbance at 570 nm.



Growth curve

Introduction:

Growth curve means graphical representation of how a particular cell line increases over time. It is important to know and record the growth characteristics of the cell line of use before starting any experiments. An alteration in cellular growth can indicate a significant problem within the cell line and if undetected can have detrimental effects on experimental results. The cellular-growth curves for distinct cell lines aiding to evaluate doubling time (DT) of population and maximum growth rate (μmax). They allow determination of the best time range for evaluating the effects of some biological compounds.

Growth curve phases:

1- lag-phase

The duration of this phase could take from a few hours up to 48 h, the time required for a cell to recover from the trypsinization, to rebuild its cytoskeleton, and to secrete an extracellular matrix that facilitates the linkage between the cells and their propagation along the substrate.

2- log-phase

All these achievements that occur in lag-phase enable the cell to enter into a new cycle. Subsequently, the cell enter into exponential growth, "log-phase", in which the cell population doubles at a characteristic rate defined as DT (characteristic for each cell line), and also the μmax value can be defined. In this phase, the effects of drugs and chemical agents that stimulate or inhibit cell growth can be studied.

3- Stationary phase

As the cell population becomes crowded when all of the substrate is occupied, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. They then enter the plateau or stationary phase, where the growth fraction drops to close to zero.

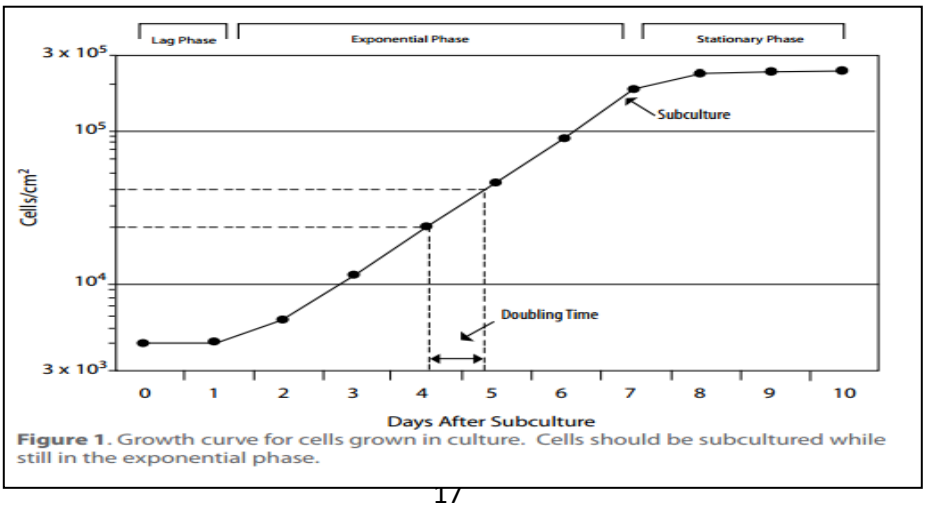


Figure 1. Growth curve for cells grown in culture. Cells should be subcultured while still in the exponential phase.

Growth curve protocol:

1-Seed suitable number of cells in each well of 6 well plat that give approximately 60% confluence after 24hr.

2-After 24hr, 48hr, 72hr and 96hr the cells harvested and preparing for growth curve protocol:

a-Collect media from the well and put it in a centrifuge tube.

b-Wash the well with 200µl trypsin, collect trypsin and put it in the centrifuge tube.

c-Add 300µl trypsin and put plate in co_2 incubator for 2 min, then put 500µl media to stop the action of trypsin.

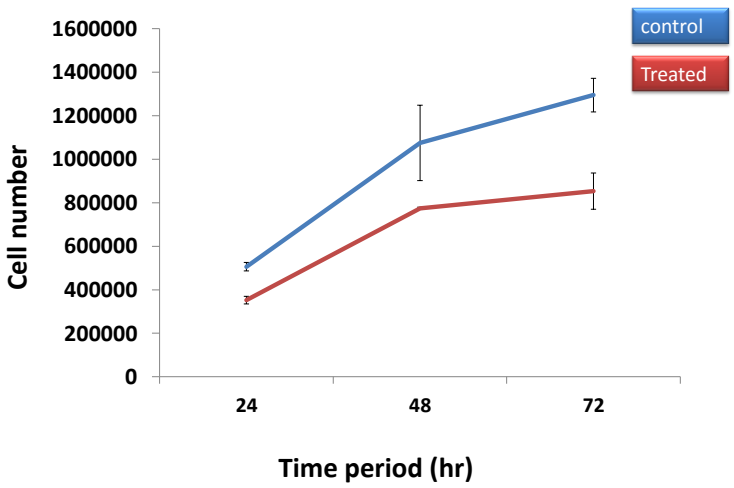
d-Use pipetting to farther loosening the cells, collect all of the well and put it in the centrifuge tube.

e-Put the centrifuge tube in centrifuge for 5 min(2500rpm/4°**C**).

f- Remove supernatant, add 1ml phosphate buffer saline and gently pipetting it.

g-Take 20 Ml from eppendorf tube, load it in hematocytometer and count the cells on microscope.

Note: Growth curve used to study the effects of drugs or anticancer agents on cancer cell proliferation rate. If you want to do this, there's another step after step2 the cells will be treated with compound to be tested.



Counting cells and cell viability assay by Trypan blue exclusion test

Introduction:

It is often necessary to count cells, for example, when plating cells for experiments such as growth curve, MTT, trypan blue, transfection and other tissue culture experiments. One method for counting cells is to use a hemocytometer. A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle. The grid has specified dimensions so that the area covered by the lines is known, which makes it possible to count the number of cells in a specific volume of solution. The most common type of hemocytometer has an "H" shape engraved in the middle that encloses two separate mirror-like polished grid surfaces and provides the cover slip mounting area (Figure 1A).

Counting cells in a hemocytometer

The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2 (Figure 1). The central counting area of the hemocytometer (Figure 1B) contains 25 large squares and each large square has 16 smaller squares. When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice (Figure 1G). Suspensions should be dilute enough so that the cells or other par For large cells, you can simply count the cells inside the four large corner squares (Figure 1C-F) and the middle one (Figure 1B). For a dense suspension of small cells you may wish to count the cells in the four outer and middle squares of the central square (Figure 1B).

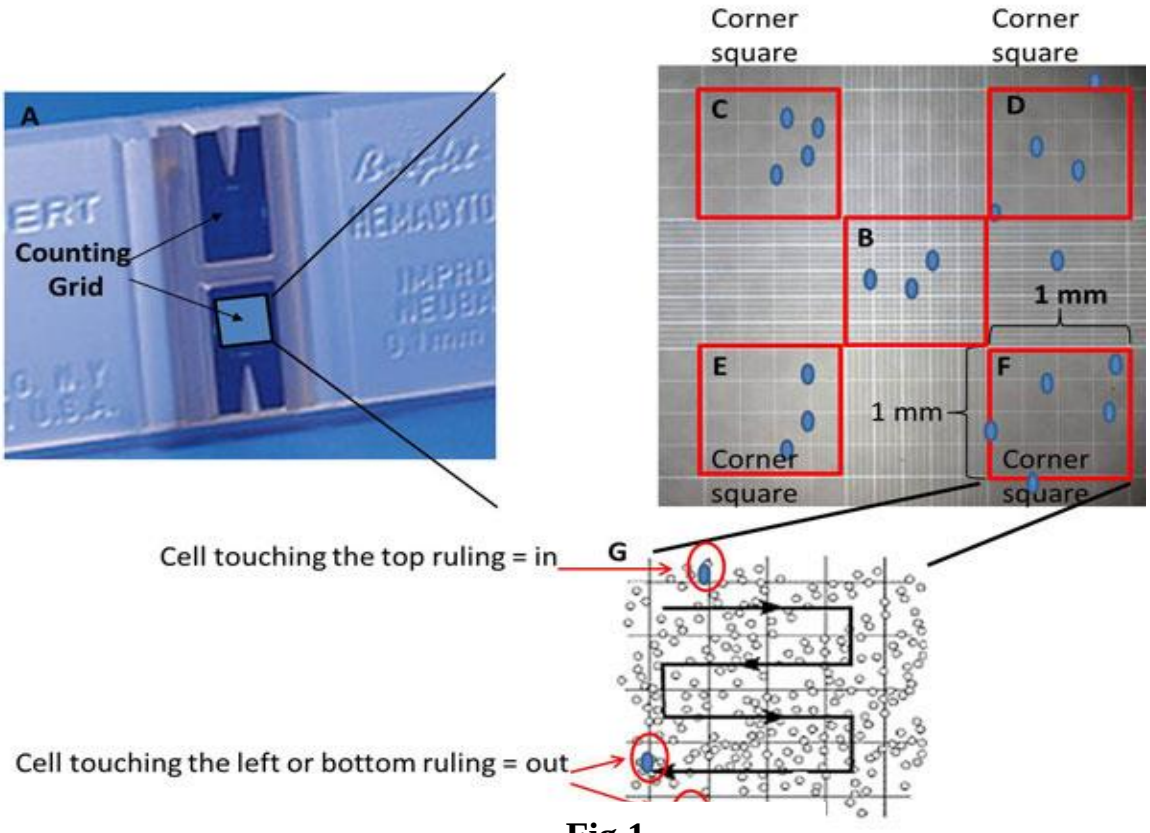


Fig 1

The volume of 1 big square is:

0,1 cm x 0,1 cm = 0,01 cm2 of area counted.

Since the depth of the chamber is 0,1mm

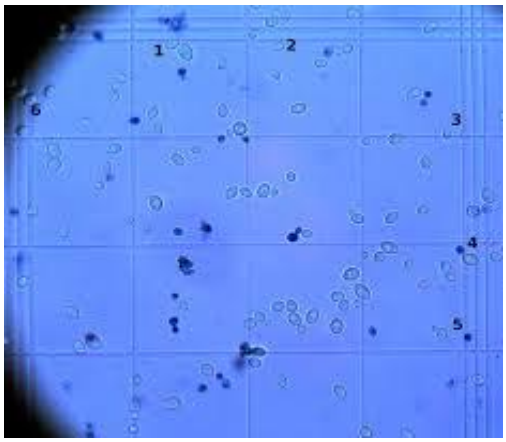
0,1 mm = 0,01 cm

0,01 cm2*0,01 cm = 0,0001 cm2 = 0,0001ml = 0,1 µl

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

Cell viability assay (Trypan blue exclusion) principle:

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.



Materials and equibments:

a) cellular dilution to measure

b) hemocytometer, or Neubabuer chamber

c) optical microscope

d) cover glass

e) pippette / micropippete with disposable tips.

f) Dilution buffer / PBS (if needed)

g) Trypan blue day

Protocol for Viable Cell Counting using Trypan Blue:

a-Collect media from the well and put it in a centrifuge tube.

b-Wash the well with 200Ml trypsin, collect trypsin and put it in the centrifuge tube.

c-Add 300Ml trypsin and put plate in co_2 incubator for 2 min, then put 500Ml media to stop the action of trypsin.

d-Use pipetting to farther loosening the cells, collect all of the well and put it in the centrifuge tube.

e-Put the centrifuge tube in centrifuge for 5 min(2500rpm/4°**C**).

f- Remove supernatant, add 1ml phosphate buffer saline and gently pipetting it.

g-take 100Ml from suspended cells, put it in eppendorf tube, put100Ml trypan blue on it, mix and leave it 3 min.

h-take 20 Ml from eppendorf tube, load it in hematocytometer and count the cells on microscope (blue and transparent cell).

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100.

Primary Culture of Specific Cell Types and the Establishment of Cell Lines

Aim :

To perform primary cell culture technique using chick embryo under aseptic condition.

Introduction:

A primary culture is that stage of the culture after isolation of the cells but before the first subculture after which it becomes a cell line.

There are four stages to consider:

(1) obtaining the sample.

(2) isolation of the tissue.

(3) dissection and/or disaggregation.

(4) culture after seeding into the culture vessel.

After isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. It appears to be essential for most normal untransformed cells, with the exception of hematopoietic cells and stem cells, to attach to a flat surface in order to survive and proliferate with maximum efficiency. Transformed cells, however, particularly cells from transplantable animal tumors, are often able to proliferate in suspension.

The enzymes used most frequently for tissue disaggregation are crude preparations of trypsin, collagenase, elastase, pronase, Dispase, DNase, and hyaluronidase, alone or in various combinations.

There are other, nonmammalian enzymes, such as Trypzean (Sigma), a recombinant, maize-derived, trypsin, TrypLE (Invitrogen), recombinant microbial, and Accutase and Accumax (Innovative Cell Technologies) also available for primary disaggregation. Crude preparations are often more successful than purified enzyme preparations. This is because the former contain other proteases as contaminants; the latter are nevertheless generally less toxic and more specific in their action.

Trypsin and pronase give the most complete disaggregation but may damage the cells. Collagenase and Dispase, in contrast, give incomplete disaggregation, but are less harmful.

Hyaluronidase can be used in conjunction with collagenase to digest the extracellular matrix, and DNase is used to disperse DNA released from lysed cells; DNA tends to impair proteolysis and promote reaggregation.

Principle :

Primary cultures are usually prepared from large tissue masses. Thus, these cultures contains a variety of differentiated cells. Embryonic tissues are preferred for primary cultures due to that the embryonic cells can be disaggregated easily and yield more viable cells. The quantity of cells used in the primary culture should be higher since their survival rate is substantially lower.

Although each tissue may require a different set of conditions, certain requirements are shared by most of them:

(1) Fat and necrotic tissues are best removed during dissection.

(2) The tissue should be chopped finely with sharp scalpels to cause minimum damage.

(3) Enzymes used for disaggregation should be removed subsequently by gentle centrifugation.

(4) The concentration of cells in the primary culture should be much higher than that normally used for subculture because the proportion of cells from the tissue that survives in primary culture may be quite low.

(5) A rich medium, such as Ham's F12, is preferable to a simple medium, such as Eagle's MEM, and if serum is required, fetal bovine often gives better survival than does calf or horse. Isolation of specific cell types will probably require selective serum-free media .

(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

Materials Required:

- 1chick embryo.
- 100 ml beaker – 1
- Dissecting tools
- Dissecting stage
- Petriplates – 2 pairs
- Culture flasks
- Trypsin
- Growth medium (DMEM).
- Phosphate buffered saline (PBS).
- Ethanol 70%

- Pipettes
- Sterile tips
- Centrifuge tubes
- Hanks BSS.
- Cups

Protocol:

1) Transfer tissue to fresh, sterile DBSS, and rinse.
2) Transfer tissue to a second dish, dissect off unwanted tissue, such as fat or necrotic material, and transfer to a third dish.
3) Chop finely with crossed scalpels into about 1-mm cubs.
4) Transfer by pipettes to a sterile centrifuge tube.
5) Allow the pieces to settle.
6) Wash by re-suspending the pieces in DBSS, allowing the pieces to settle, and removing the supernatant fluid. Repeat this step two more times.
7) Transfer the pieces to a culture flask “20-30” pieces.
8) Remove most of the fluid, and add 1 ml growth medium.
9) Cap the flask, and place it in an incubator 37C for 18-24 hrs.
10) If the pieces have adhered, the medium volume may be made up gradually over the next 3-5 days to 10 ml and then change weekly.
11) When you observe growth in culture , transfer the tissue into fresh culture vessel.
12) Subculture if necessary.

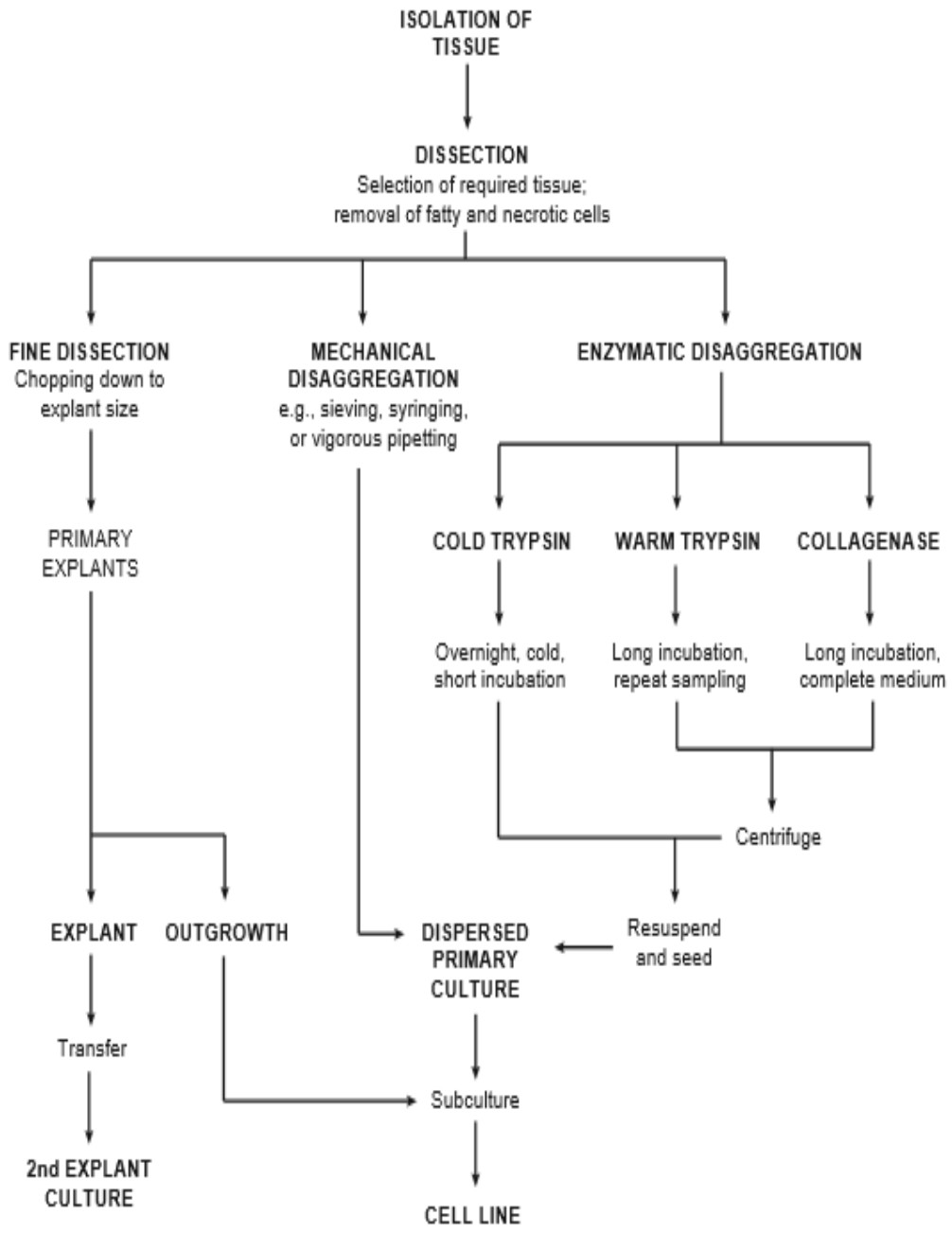


Fig. 11.5. Options for Primary Culture. Multiple paths to obtaining a cell line; center and left, by mechanical disaggregation, right, by enzymatic disaggregation. An explant may be transferred to allow further outgrowth to form, while the outgrowth from the explant may be subcultured to form a cell line.

Migration assay

Introduction:

The *in vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration *in vitro.* It is particularly suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration. Farther more it's used for screening the effects of pharmaceutical substances or genetic modifications on mammalian cells. This method is based on the observation that, upon creation of a new artificial gap, so called "scratch", on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the "scratch" until new cell–cell contacts are established again.

Objectives:

1-To be able to do scratch assay.

2-To be able to adjusting microscope and take an images.

3- Analysis of the image and quantify the migration rate using Image analysis software.

Principle

The basic steps involve creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells.

Materials and equipment's :

1-6cm dishes
2-1ml sterile tips
3-Phosphate-buffered saline (PBS)
4-RPMI 1640 media
5-Hela cells
6-marker
7-Inverted microscope with camera
8-Image analysis software.

Protocol:

1-Drow straight lines underside of the 6cm plate so that the spacing between them is small.

2-Sead 200.000 Hela cells in 6cm plate.

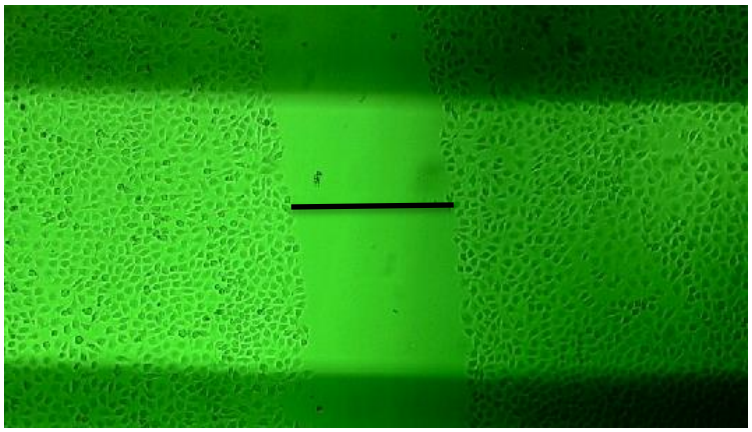
3-After 72hr the confluence of the plate is approximately 90%, then gently and slowly scratch the monolayer with a new 1 ml pipette tip across the center of the plate perpendicular to the straight lines.

4-Remove the media and add new media to the plate.

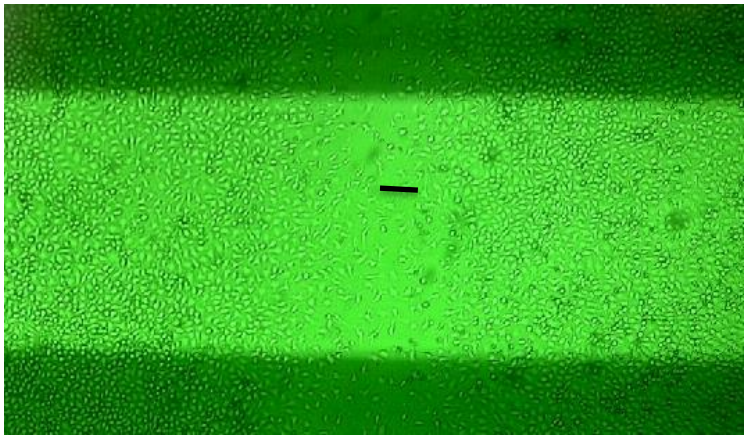
5-Take photos for the plates on a microscope. Set the same configurations (10X) of the microscope when taking pictures for different views of the plate (at least take photos to 10 region between straight lines) the photos taken at 0 time, 24hr,48hr and 72hr to each control and treated plates.

6-The gap distance can be quantitatively evaluated using ImageJ software.

At 0 Time



After 48hr



CRYOPRESERVATION AND THAWING OF CELLS

Aim :

To preserve the cells in viable condition for future works by using proper preservative.

Introduction:

A variety of primary cells, cell strains and established cell lines have been shown to survive when stored at -65ºC or below without any change of properties. The critical points in the technique are :

1) Slow freezing,

2) Rapid thawing,

3) Use of 5-20 % (v/v) glycerol or 5-10% (v/v) dimethyl sulfoxide (DMSO) in the freezing and storage,

4) Storage at temperatures below -70ºC.

Once at ultralow temperatures, the cells are biologically inert and can be preserved for years.

Advantages of cryopreservation:

1) Reduce the risk of microbial contamination.
2) Reduce the risk of cross contamination with other cell lines.
3) Reduce the risk of genetic drift and morphological changes.
4) Reduce costs (consumable and staff time).

Common cryoprotectants:

The cell suspension is frozen in the presence of a cryoprotectant such as glycerol or dimethyl sulfoxide (DMSO).Of these two, DMSO appears to be the more effective, possibly because it penetrates the cell better than glycerol. Concentrations of between 5% and 15% have been used, but 7.5% or 10% is more usual.

There are situations where DMSO may be toxic or induce cells to differentiate after thawing. Glycerol may be sterilized by autoclaving for 15minutes and should be stored in small aliquots to prevent introduction of contaminants. DMSO should be

sterilized by filtration with a 0.2micron nylon syringe filter and stored at -20C in small, single-use sealed aliquots.

Air oxidation of DMSO is relatively rapid and these products are toxic to cells. DMSO should be allowed to come into contact with the skin as it is rapidly absorbed and is a reported neurotoxin.

Materials:

- Cultured cells
- Culture medium (DMEM)
- Cryovials or glass ampoules.
- Cryogenic freezer (-150C).
- Cryoprotective agent(DMSO).
- -80 C freezer.

Principle:

During Preservation metabolic activity of the cells get inactivated or rate of cell division get slow by the addition of cryoprotectant i.e. Dimethyl sulfoxide (DMSO) and Glycerol in the appropriate concentration with liquid nitrogen, this type of preservation is called Cryopreservation. With which cells will be alive for many number of years.

Protocol:

1.Grow the culture up to the late log phase, and if you are using a monolayer, trypsinize and count the cells, If you are using a suspension, count and centrifuge the cells.

2.Resuspend at 2×106 to 2×107 cells/mL.

3. Dilute one of the cryoprotectants in growth medium to make freezing medium:

(a) Add dimethyl sulfoxide (DMSO) to between 10% and 20%.

(b) Add glycerol to between 20% and 30%.

4. Dilute the cell suspension 1:1 with freezing medium to give approximately 1×106 to 1× 107 cells/mL and 5% to 10% DMSO (or 10–15% glycerol). It is not necessary to place ampoules on ice in an attempt to minimize deterioration of the cells. A delay of up to 30 min at room temperature is not harmful when using DMSO and is beneficial when using glycerol.

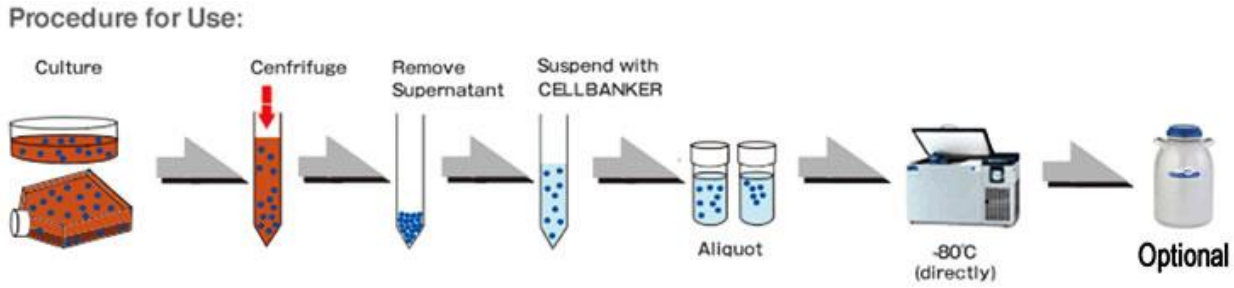
5. Dispense the cell suspensions into prelabeled ampoules, and cap the ampoules with sufficient torsion to seal the ampoule without distorting the gasket.

6. Place the ampoules on canes for canister storage , or leave them loose for drawer storage.

7.Freeze the ampoules at 1◦C/min by one of the methods described above. With the insulated container methods, this will take a minimum of 4 to 6 h after placing them at −70◦C if starting from a 20◦C ambient temperature, but preferably leave the ampoules in the container at −70◦C overnight.

8. When the ampoules have reached −70◦C or lower, check the freezer record before removing the ampoules from the −70◦C freezer or controlled rate freezer, and identify a suitable location for the ampoules. 10. Transfer the ampoules to the liquid N2 freezer, preferably not submerged in the liquid, placing the cane and tube into the predetermined canister or individual ampoules into the correct spaces in the predetermined drawer. This transfer must be done quickly (<2 min), as the ampoules will
reheatatabout10◦C/min,andthecellswilldeteriorateraplidlyifthetemperaturerisesabove−50◦C.

9. When the ampoules are safely located in the freezer, complete the appropriate entries in the freezer index.



Thawing of storage cells

When required, cells are thawed and reseeded at a relatively high concentration to optimize recovery. The ampoule should be thawed as rapidly as possible to minimize intracellular ice crystal growth during the warming process. This can be done in warm water, in a bucket, or in a water bath.

The cell suspension should be diluted slowly after thawing as rapid dilution reduces viability. This gradual process is particularly important with DMSO, with which sudden dilution can cause severe osmotic damage and reduce cell survival by half.

Protocol:

1.Check the index for the location of the ampoule to be thawed.

2. Collect all materials, prepare the medium, and label the culture flask.

3. Retrieve the ampoule from the freezer, check from the label that it is the correct one, and, if it has not been submerged in liquid nitrogen, place it in sterile water at 37◦C in a beaker or water bath. If possible, avoid getting water up to the cap as this will increase the chance of contamination. A heating block is useful for this, though heat transfer may be slower.

4. When the ampoule has thawed, double-check the label to confirm the identity of the cells; then swab the ampoule thoroughly with 70% alcohol, and open it in a laminar-flow hood.

5. Transfer the contents of the ampoule to a culture flask with a 1-mL pipette of pipettor with filter tip. 6. Add medium slowly to the cell suspension: 10 mL over about 2 min added drop wise at the start, and then a little faster, gradually diluting the cells and cryoprotectant. For cells that require centrifugation to remove the cryoprotectant: (a) Dilute the cells slowly, as in step 6, but in a centrifuge tube or universal container. (b) Centrifuge them for 2 min at 100 g . (c) Discard the supernatant medium with the cryoprotectant. (d) Resuspend the cells in fresh growth medium. (e) Seed flask for culture.

7. The dregs in the ampoule may be stained with Naphthalene Black or Trypan Blue to determine cell viability .

8. Check after 24 h:

(a) For attached monolayer cells, confirm attachment and try to estimate percentage survival based on photographs of cells at the expected density(cells/cm2)with full survival .

(b) For suspension-growing cells, check appearance (clear cytoplasm, lack of granularity), and dilute to regular seeding concentration. This can be made more precise if the cells are counted and an estimate of viability is made, in which case the cells can be diluted to the regular seeding concentration of viable cells.

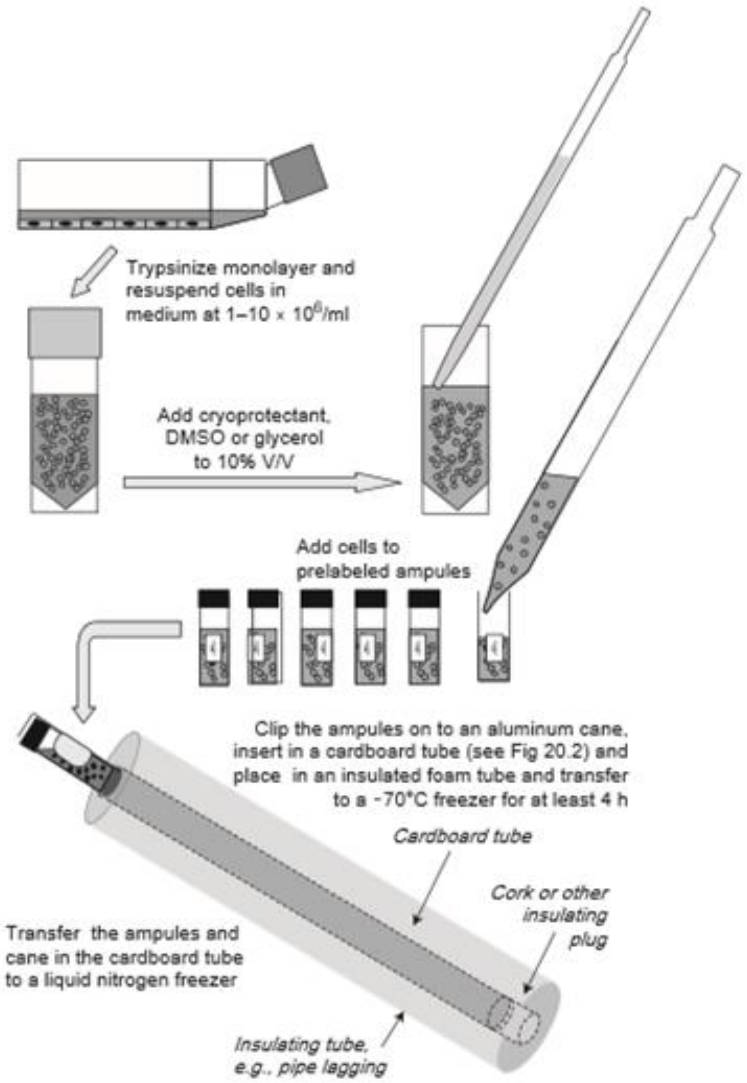


Fig. 19.8. Freezing Cells. Trypsinized cells, in medium with cryoprotectant, aliquoted into ampoules, which are then clipped on to an aluminum cane, inserted into a cardboard tube, and inserted into an insulated tube. The tube and contents are placed at −70°C or −80°C for 4 h or overnight, before transferring the cardboard tube containing the ampoules to a liquid nitrogen freezer with canister storage (*see* Fig. 19.7*a*).

Colony formation or plating efficiency

Introduction:

Colony formation at low cell density, or plating efficiency, is the preferred method for analyzing cell proliferation and survival. This technique reveals differences in the growth rate within a population and distinguishes between alterations in the growth rate (colony size) and cell survival (colony number). To assay the growth-promoting ability of a test medium or serum. Heterogeneity in clonal growth rates reflects differences in the capacity for cell proliferation between lineages within a population. When cells are plated out as a single-cell suspension at low cell densities (2–50 cells/cm2), they grow as discrete colonies. The number of these colonies can be used to express **the plating efficiency.**

$$\frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100 = \text{Plating efficiency}$$

If it can be confirmed that each colony grew from a single cell, then this term becomes the **cloning efficiency**. Seed the cells at low density, and incubate until colonies form stain and count the colonies

Materials and methods:

Materials

Sterile:

Culture of adherent cells
Growth medium.....400 mL
Trypsin, 0.25%, crude.....10 mL

Petri dishes, 6 cm.....20
Tubes, or universal containers, for dilution.....20

Nonsterile:

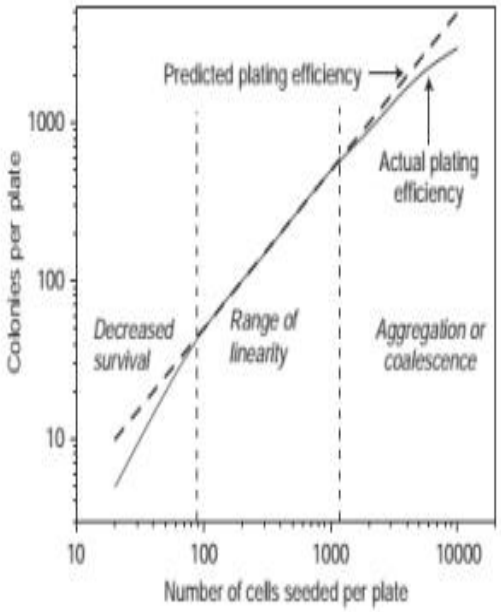
- Hemocytometer or electronic cell counter
- Fixative: anhydrous methanol.....100 mL
- D-PBSA.....200 mL
- Stain: Crystal Violet.....100 mL
- Filter funnel and filter paper (to recycle the stain)

Protocol:

Procedure

1. Trypsinize the cells (see Protocol 12.3) to produce a single-cell suspension.
2. While the cells are trypsinizing,
   (a) Number the dishes on the side of the base.
   (b) Measure out medium for the dilution steps (Fig. 20.14). There should be more than enough medium for three replicates at each dilution.
3. When the cells round up and start to detach:
   (a) Disperse the monolayer in medium containing serum or a trypsin inhibitor.
   (b) Count the cells.
   (c) Dilute the cells to:
      (i) 2×10^4/mL for two 25-cm^2 flasks for routine maintenance.
      (ii) 2×10^3 cell/mL as top concentration for subsequent dilutions.
      (iii) Five further dilutions from (ii) to give 200, 100, 50, 20, and 10 cells/mL.
4. Seed the Petri dishes with 5 mL medium containing cells at each of the five concentrations in (iii). Seed two 6-cm Petri dishes at 2×10^3 cells/mL to act as controls in case the cloning is unsuccessful (to prove that there were cells present in the top dilution, at least).
5. Gas the flasks with 5% CO_2 and take to incubator.
6. Put the Petri dishes in a transparent plastic box and place in a humid CO_2 incubator, preferably one with limited access and reserved for cloning.
7. Incubate the dishes until colonies are visible to the naked eye (1–3 weeks).
8. Stain the colonies with Crystal Violet:
   (a) Remove the medium from the dishes.
   (b) Rinse the cells with D-PBSA, and discard the rinse.
   (c) Add 5 mL fresh D-PBSA and then add 5 mL methanol with gentle mixing (avoid colonies detaching).
   (d) Replace the 50:50 D-PBSA:methanol mixture with 5 mL fresh methanol, and fix the cells for 10 min.
   (e) Discard the methanol, and add Crystal Violet, neat, 2 to 3 mL per 6-cm dish, making sure that the whole of the growth surface is covered.
   (f) Stain for 10 min.
   (g) Remove the stain, and return it to the stock bottle of stain via a filter.
   (h) Rinse the dishes with water and allow to dry.
9. Count the colonies in each dish, excluding those below 50 cells per colony. Magnifying viewers can make counting the colonies easier.

Note. It will be necessary to define a threshold above which colonies will be counted. If the majority of the colonies are between a hundred and a few thousands, then set the threshold at 50 cells per colony. In practice, this is a fairly natural threshold when counting by eye. However, if the colonies are very small (<100 cells), then set the threshold at 16 cells per colony. Below 16 cells, equivalent to 4 cell consecutive divisions, it would be hard to presume continued cell proliferation.



Linearity of Plating Efficiency. If plating efficiency remains constant over a wide range of cell concentrations, the curve is linear (dashed line), whereas if there is poor survival at low densities or aggregation or coalescence at high densities, plating efficiency decreases (solid line)

Clonogenic assay

Introduction:

Short-term tests are convenient and usually quick and easy to perform, they reveal only cells that are dead at the time of the assay. Frequently, however, cells that have been subjected to toxic influences (e.g., irradiation, environmental toxins, and antineoplastic drugs) show an effect several hours, or even days, later. The nature of the tests required to measure viability in these cases is necessarily different because, by the time the measurement is made, the dead cells may have disappeared or some resistant cells may have recovered. Therefore long-term tests are used to demonstrate survival rather than short-term toxicity. Clonogenic assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents.

Principle:

Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 1–3 weeks. Colonies are fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted using a stereomicroscope.

Materials and Reagents

1. Cell culture medium
2. Phosphate buffered saline (PBS)
3. Fetal bovine serum (FBS)
4. Trypsin/ EDTA
5. Crystal violet
6. Methanol
7. Glacial acetic acid
8. Fixation solution
9. Colony fixation solution
10. Crystal violet solution

Equipment

1. Cell culture petri dishes or six-well plates.
2. Hemocytometer
3. Microscope
4. Incubator

Reagent:

1. Colony fixation solution
   Acetic acid/methanol 1:7 (vol/vol)
2. Crystal violet 0.5% solution

Procedure

A. **Assay setup:**

Cell can be plated either before or after the treatment.

1. Plating before treatment:

a. Harvest cells and plate an appropriate number of cells per dish or per well on a 6-well plate, at least in duplicate. The number of cells for seeding should be determined by the aggressiveness of the treatment. Incubate cells for a few hours in a CO_2 incubator at 37 °C and allow them to attach to the plate/dish.

b. Treat the cells as necessary with the certain drug, and keep control cells without treatment.

c. Incubate the cells in a CO_2 incubator at certain time period, then harvest cells and re- plating it in at low number.

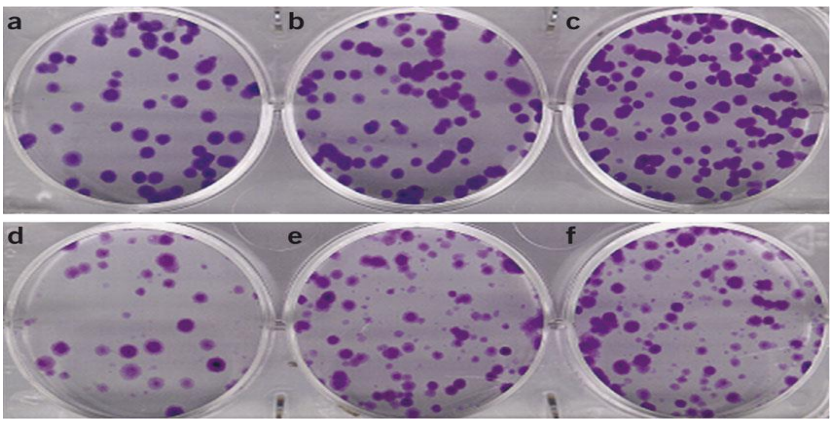
d. Incubate the cells in a CO_2 incubator at 37 °C for 1-3 weeks until cells in control plates have formed colonies with substantially good size (50 cells per colony is the minimum for scoring).

B. Fixation and staining:

1. Remove medium, and then rinse cells with 10 ml PBS.
2. Remove PBS and add 2-3 ml of fixation solution and leave the dishes/plates at room temperature (RT) for 5 min.
3. Remove fixation solution.
4. Add 0.5% crystal violet solution and incubate at RT for 2 h.
5. Add 10 ml medium with 10% FBS, and detach the cells by pipetting.
6. Remove crystal violet carefully and immerse the dishes/plates in tap water to rinse off crystal violet.
7. Air-dry the dishes/plates on a table cloth at RT for up to a few days.

C. Data analysis:

1. Count number of colonies with a stereomicroscope.
2. Calculate plating efficiency (PE) and surviving fraction (SF).
   PE = no. of colonies formed/ no. of cells seeded x 100%
   SF = no. of colonies formed after treatment/ no. of cells seeded x PE



Anchorage independence assays

Introduction:

Neoplastic transformation occurs via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth. Anchorage-independent growth is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells.

Principle:

The soft agar colony formation assay is a common method to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after 3-4 weeks by manual counting of colonies. This method depends on making a base agar 0.5 % and top agar having cells. Then plate your cells in the top agar and allow them to make colonies in the agar.

Material:

0.7 and 1 % agar

2x media

10 ml tubes

Trypsinze cells

6 cm dishs

Protocol

1. Preparation of Materials and Reagents

1. Label each well of a tissue culture treated 6-well plate appropriately for each cell line or condition being investigated.

2. Prepare 2x cell culture medium by dissolving 1 g of powder medium and 0.2 g of sodium bicarbonate in de-ionized water to a final volume of 50 ml.

3. Pass this medium through a 0.2 μm filter to sterilize.

4. Add additional components needed for normal culture of the cell line of interest. For example, grow CMT 167 cell line in RPMI 1640 medium supplemented with 10%

FBS and 1% penicillin/streptomycin solution. Warm medium to 37 °C in hot water bath prior to use.

5. Prepare 1x cell culture medium separately as you would for normal cell culture of the cell line of interest. 6. Prepare 1% noble agar by adding 1 g of noble agar to 100 ml of deionized water. NOTE: Noble agar will not dissolve completely with agitation alone.

7. Prepare 0.6% noble agar by adding 0.6 g of noble agar to 100 ml of de-ionized water. Both agar solutions can be made in 100 ml glass bottles with closable lids for long-term storage.

8. Autoclave the noble agar mixtures to sterilize. These mixtures can be made in advance and stored at 4 °C but should be heated again at the time of the experiment until agar has completely dissolved.

9. Prepare nitroblue tetrazolium chloride solution by making a 1 mg/ml stock solution in 1x PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 in H2O to final volume of 1,000 ml). This will be used at the end of the experiment to stain the colonies.

2. Plating of Bottom Layer of Agar

1. Loosen the cap on the bottle of 1% noble agar and microwave for about 1-2 min. While heating in a microwave, monitor the solution closely to avoid boiling over. Continue heating, while mixing intermittently, until agar is completely dissolved and the solution is clear. NOTE: Use heat-resistant gloves to handle flask after heating. Failing to do so may cause burn or serious injury.

2. Place melted agar solution and pre-warmed 2x culture medium in an ice bucket filled with hot tap water (42 °C). Also place a 50 ml conical tube in a tube holder in the ice bucket with hot water. Transfer bucket to cell culture hood for subsequent steps.

3. For the bottom layer of agar, you will need 1.5 ml of a mix of agar and medium per well of a 6-well plate.

4. To ensure an adequate amount of the mixture, prepare a total of 12 ml for each 6-well plate.

5. Start by adding 6 ml of culture medium to the 50 ml conical tube and then 6 ml of the 1% noble agar solution.

6. Invert the conical tube several times to mix. Working at a brisk pace will prevent premature hardening of the soft agar.

7. Draw up approximately 5.5 ml of mixture into a 5 ml serological pipette.

8. Allow the air bubbles to rise to top of pipette column before depositing 1.5 ml of this mixture into each well. Use caution to avoid deposition of any air bubbles into the plate wells.

9. Cover the plates and allow agar mixture to solidify at room temperature, in cell culture hood, for 30 min.

3. Plating the Upper Layer of Agar Containing Cells

1. Once the lower layer of agar has solidified, begin preparation of the upper layer.

2. First, harvest cells by trypsinization and dilute them 1:5 in culture medium (e.g. for 1 ml of trypsin, add 4 ml of medium) into a 15 ml conical tube.

3. Count cells and calculate the number of cells needed per well to prepare a final cell suspension at this time. This number will vary depending on cell type. Use 5,000 cells/well as a starting point and adjust as needed. For this cell number, you would prepare a cell suspension of 6,667 cells/ml (i.e. each well will receive 0.75 ml of this suspension and 0.75 ml of agar for a total volume of 1.5 ml; the concentration of cells will also be diluted 1:2 for a final total cell count of 5,000).

4. The volume of cell suspension needed per well of a 6-well plate will again be 1.5 ml. Prepare additional cell suspension totaling 12 ml per 6- well plate.

5. Melt 0.6% agar solution in a microwave as above and place into ice bucket containing hot water along with a 50 ml conical tube in a tube holder and the final cell suspension from Step 3.3.

6. Transfer the ice bucket with melted 0.6% agar to cell culture hood for subsequent steps.

7. Mix 0.6% agar and cell suspension in a 1:1 ratio, preparing a total volume of 12 ml per 6-well plate. 1.5 ml will be required per well but extra should be made as above.

8. Pipette 6 ml of cell suspension into the 50 ml conical tube.

9. Then, add 6 ml of 0.6% agar to the tube. NOTE: The temperature of this mixture must be kept around 42 °C to avoid premature hardening and to maximize cell survival.

10. Working quickly, pipette this mixture 2-3x to distribute cells, then draw up 5.5 ml of mixture into a 5 ml serological pipette.

11. Allow any air bubbles to rise to top of pipette column before depositing 1.5 ml of this mixture into each well. Use caution to avoid deposition of any air bubbles into the plate wells.

12. Allow cell/agar mixture to solidify at room temperature, in cell culture hood, for 30 min before placing into a 37 °C humidified cell culture incubator.

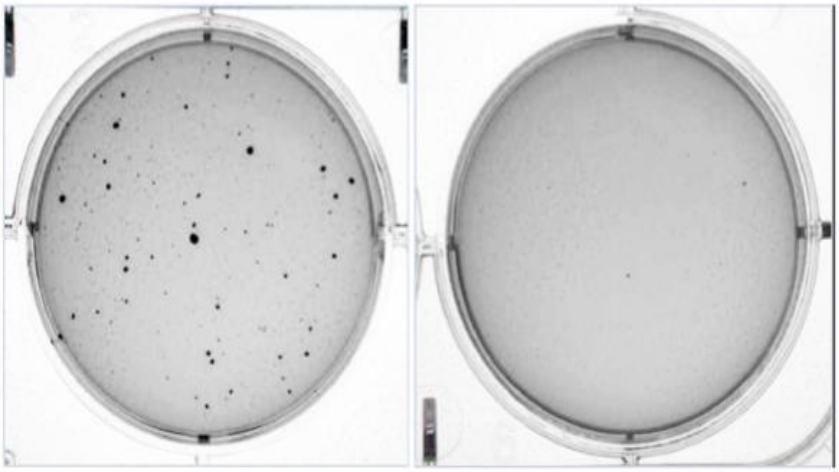
13. The time required for adequate colony formation varies for each cell line, typically around 21 days.

14. A layer of growth medium should be maintained over the upper layer of agar to prevent desiccation. 100 µl of medium added twice weekly is sufficient for this purpose.

4. Staining the Plates and Counting Colonies

1. Stain cells by adding 200 µl of nitroblue tetrazolium chloride solution per well and incubating plates overnight at 37 °C.

2. Once colonies are stained, take photographs of wells using an imager and count colonies using image analysis software.



CMT167 LNCX/LPCX CMT167 LL Wnt7A/Fzd9

Figure 4: Soft Agar Colony Formation Assay of vector-expressing CMT167 LNCX/LPCX cells and CMT167 LL Wnt7a/Fzd9 overexpressing cells. Vector-expressing CMT167 LNCX/LPCX cells and CMT167 LL Wnt7A/Fzd9 overexpressing cells were plated in a soft agar colony formation assay per protocol described above. CMT167 LL Wnt7A/Fzd9 cells showed a marked decrease in colony formation. Representative wells showing colonies for each cell line. Picture of wells are representative of three independent experiments.

Apoptosis detection by fluorescent dyes

INTRODUCTION

Nucleus Staining Fluorescent dyes with aromatic amino or guanidine groups, such as propidium iodide (PI), ethidium bromide (EB), diaminophenylindole (DAPI), acridine orange (AO), and Hoechst dyes, interact with nucleotides to emit fluorescence. EB and PI molecules intercalate inside the DNA double helix. DAPI and Hoechst dye molecules attach at the minor groove of the DNA double helix. On the other hand, AO can form complexes with either double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can intercalate with three base pairs of double-stranded DNA to emit green fluorescence with the maximum wavelength at 526 nm. One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure that emits red fluorescence with the maximum wavelength at 650 nm. These fluorescent dyes, except for the Hoechst dyes, are impermeable through the cell membranes of viable cells, and can be used as fluorescent indicators of dead cells. Hoechst dyes are positively charged under physiological conditions and can pass through viable cell membranes.

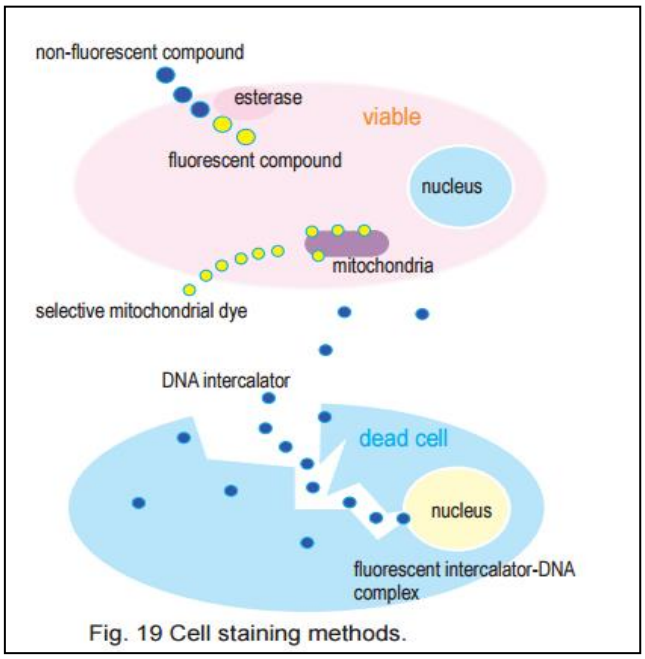


Fig. 19 Cell staining methods.

Materials:

1-Cells growing in culture

2-Phosphate-buffered saline (PBS)

3-Hoechst® 33342, trihydrochloride, trihydrate

4-Fluorescence microscope

Protocol

Preparing Hoechst® dye stock solution

1. Prepare the Hoechst® dye stock solution by dissolving the contents of one vial (100 mg) in 10 mL of deionized water (diH_2O) to create a 10 mg/mL (16.23 mM) solution. Note: Hoechst® dye has poor solubility in water, so sonicate as necessary to dissolve. The 10 mg/mL Hoechst® stock solution may be stored at 2–6°C for up to 6 months or at ≤–20°C for longer periods.

Labeling cells

1. Culture cells in an appropriate medium and vessel for fluorescence microscopy.

2. Prepare the Hoechst® staining solution by diluting the Hoechst® stock solution 1:2,000 in PBS.

3. Remove the medium.

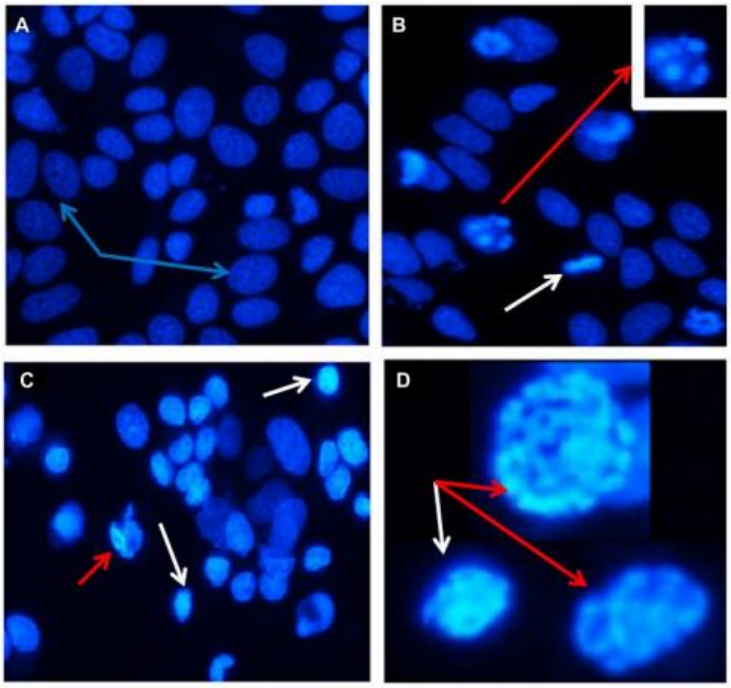
4. Add sufficient staining solution to cover the cells.

5. Incubate for 5–10 minutes, protected from light.

6. Optional: You may image directly in the staining solution, if you wish.

	7. Remove the staining solution.
	8. Wash the cells 3 times in PBS.
	9. Image the cells.

Result:



THE END