Lab. 1: Cell Culture Equipment

1. Tissue Culture Hood

— Laminar flow hoods. There are two types of laminar flow hoods, vertical and horizontal. The vertical hood is also known as a biology safety cabinet.

— Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particle) filter that removes particulates from the air.

— The hoods are equipped with a short-wave UV light that can be turned on for a few minutes to sterilize the surfaces of the hood, but be aware that only exposed surfaces will be accessible to the UV light.

Preparation of a Tissue Culture

—

2. Cell Culture: Incubator

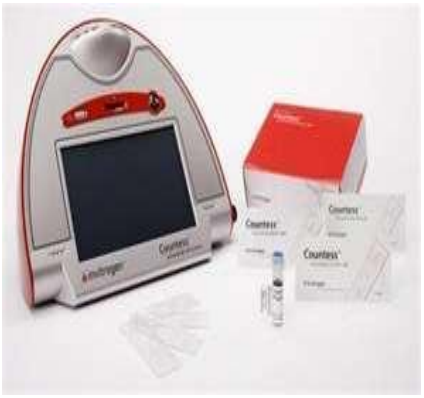
CO2 Incubators: The cells are grown in an atmosphere of 5-10% CO2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. Culture flasks should have loosened caps to allow for sufficient gas exchange. Cells should be left out of the incubator for as little time as possible and the incubator doors should not be opened for very long. The humidity must also be maintained for those cells growing in tissue culture dishes so a pan of water is kept filled at all times

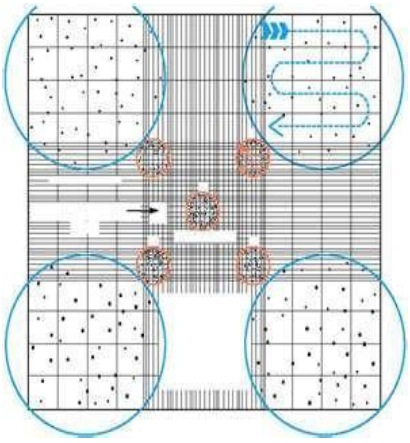
3. Water Bath

4. Centrifuge

5. Refrigerator and freezer

6. Cell Counter (eg. CountessR Automated Cell Counter or hemacytometer)





7. Cell Culture Microscope

— Microscopes. Inverted phase contrast microscopes are used for visualizing the cells. Microscopes should be kept covered and the lights turned down when not in use.

Lab. 2: Cell culture environment

Physic-chemical environment

- Growth media (pH, osmotic pressure, O_2 and CO_2 tension)
- Temperature

1- Physiological environmental

- Hormone and nutrient concentrations

Cell culture media

The culture medium is the most important component of the culture environment because it provides:

- Necessary nutrients
- Growth factors
- Hormones for cell growth
- Regulating the pH of culture
- Osmotic pressure of the culture

.

Commonly used Medium:

GMEM, EMEM, DMEM, RPMI etc.

Media is supplemented with

- Antibiotics (penicillin, streptomycin)
- BSA
- Na-bicarbonate
- L-glutamate
- Na-pyruvate
- HEPES
- Growth factors etc

Cell Culture Media and Solutions

Antimycotic/Antibiotic Media
To 1 liter of sterile RPMI 1640 with 2 mM L-glutamine, add:
- 165.0 mL fetal bovine serum, heat-inactivated
- 12.0 mL 200 mM (100X) L-glutamine
- 12.0 mL antimycotic/antibiotic (100X), liquid, Gibco, Cat. No. 600-5240AG
- Filter-sterilize through a 0.22-µm cellulose acetate filter and store up to 2 weeks at 4°C.

Cyclosporin Media: (100 mL)
To 100 mL of growth media, add 1.0 mL 100X Cyclosporin A

Cyclosporin A Media: (1 µg/mL)
To 100 mL of growth media add 2 mL of 100X Cyclosporin A.

Cyclosporin A: (100 mL)
Dissolve 1 mg CSA in 0.1 mL ethanol in a sterile 15-mL centrifuge tube with a small magnetic stirrer. Add 0.02 mL (= 20 mL) of Tween 80 and mix well. While continually stirring, add 1 mL RPMI drop by drop. Bring to a final volume of 100 mL with RPMI. Filter-sterilize with a 0.22-mm filter. Store at 4°C for up to 4 months.

Freezing Media: (1 liter)
Prepare a 1-liter volume and divide into 25–50 mL centrifuge tubes containing 40 mL each.

Store the tubes at –80°C for up to 1 year.
700 mL RPMI-1640 with 2 mM L-Glutamine
200 mL fetal bovine serum (FBS)
100 mL dimethyl sulfoxide (DMSO, Sigma)
1000 mL total volume

Filter-sterilize media and FBS with a 0.22-mm cellulose acetate filter. Do not filter DMSO, it will dissolve the cellulose acetate membrane.

Growth Media: (600 mL)

To 500 mL of sterile RPMI 1640 with 2 mM L-glutamine, add:
90.0 mL FBS
6.0 mL 200 mM (100X) L-glutamine
0.6 mL 50 mg/mL gentamicin reagent

Filter-sterilize through a 0.22-mm filter and store up to 2 weeks at 4°C.

Growth Media: (1 liter)

To 1 liter of sterile RPMI 1640 with 2 mM L-glutamine, add:
165.0 mL fetal bovine serum, heat-inactivated at 50°C–60°C for one and half hour.
12.0 mL 200 mM (100 X) L-glutamine
1.2 mL 50 mg/mL gentamicin reagent

Filter-sterilize through a 0.22-mm cellulose acetate filter and store up to 2 weeks at 4°C.

Wash Media: (1 liter)

To 1 liter of sterile RPMI 1640 with 2 mM L-glutamine, add:
10.0 mL 2.5 M (100X) HEPES buffer
1.2 mL 50 mg/mL gentamicin reagent

Filter-sterilize through a 0.22-mm cellulose acetate filter and store up to 2 weeks at 4°

Lab. 3: Contamination of cell culture

Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions. But in practice it refers to the culturing of cells derived from animal cells, Cell culture was first successfully undertaken by Ross Harrison in 1907.

Major development's in cell culture technology

1. First development was the use of antibiotics which inhibits the growth of contaminants.
2. Second was the use of trypsin to remove adherent cells to subculture further from the culture vessel
3. Third was the use of chemically defined culture medium.

Why is cell culture used for?

Areas where cell culture technology is currently playing a major role.

1. Model systems for

Studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies

2. Toxicity testing

   Study the effects of new drugs

3. Cancer research

   Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells.

4. Virology

Cultivation of virus for vaccine production, also used to study there infectious cycle.

5. Genetic Engineering

Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

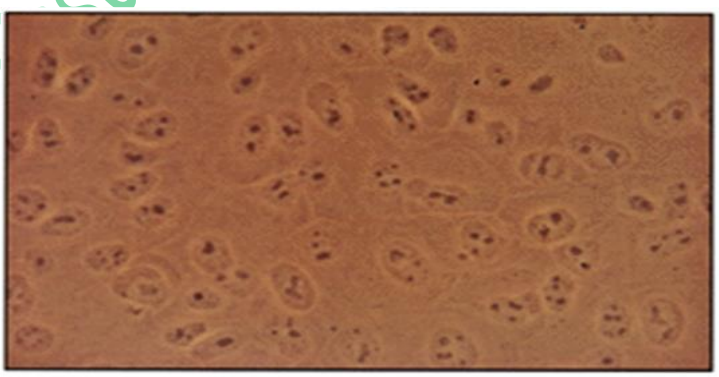
6. Gene therapy

Cells having a functional gene can be replaced to cells which are having non-functional gene.

Contaminant's of cell culture

Cell culture contaminants of two types

1. Chemical-difficult to detect caused by endotoxins, plasticizers, metal ions or traces of disinfectants that are invisible
2. Biological-cause visible effects on the culture they are mycoplasma, yeast, bacteria or fungus or also from cross-contamination of cells from other cell lines.



Effects of Biological Contamination's

1. They competes for nutrients pwith host cells
2. Secreted acidic or alkaline by-products ceses the growth of the host cells
3. Degraded arginine & purine inhibits the synthesis of histone and nucleic acid
4. They also produces H2O2 which is directly toxic to cells

Detection of contaminants

1. In general indicators of contamination are turbid culture media, change in growth rates, abnormally high pH, poor attachment, multi-nucleated cells, graining cellular appearance, vacuolization, inclusion bodies and cell lysis
2. Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH)
3. Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst .
4. Mycoplasma also detected by enzyme immunoassay by specific antisera or monoclonal abs or by PCR amplification of mycoplasmal RNA
5. The best and the oldest way to eliminate contamination is to discard the infected cell lines directly.

Basic aseptic conditions

1. If working on the bench use a Bunsen flame to heat the air surrounding the Bunsen
2. Swab all bottle tops & necks with 70% ethanol

3. Flame all bottle necks & pipette by passing very quickly through the hottest part of the flame
4. Avoiding placing caps & pipettes down on the bench; practice holding bottle tops with the little finger
5. Work either left to right or vice versa, so that all material goes to one side, once finished
6. Clean up spills immediately & always leave the work place neat & tidy.

Safety aspect in cell culture

1. Possibly keep cultures free of antibiotics in order to be able to recognize the contamination.
2. Never use the same media bottle for different cell lines. If caps are dropped or bottles touched unconditionally touched, replace them with new ones.
3. Necks of glass bottles prefer heat at least for 60 sec at a temperature of 200 C.
4. Switch on the laminar flow cabinet 20 min prior to start working.
5. Cell cultures which are frequently used should be subcultered & stored as duplicate strains.

Lab. 4&5: Primary Culture

primary cell culture refers to the cells the first time they are placed in culture.Once these cells have been subcultured are no longer primaries and should not be described as primary culture Primary cell cultures are derived from rat, mouse and human in the Lab.

Properties of primary culture

1. Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture
2. Primary cells have a finite life span
3. Primary culture contains a very heterogeneous population of cells
4. Sub culturing of primary cells leads to the generation of cell lines
5. Cell lines have limited life span, they passage several times before they become senescent
6. Cells such as macrophages and neurons do not divide in vitro so can be used as primary cultures
7. Lineage of cells originating from the primary culture is called a cell strain

Types of cells

On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three.

1. Epithelial like-attached to a substrate and appears flattened and polygonal in shape
2. Lymphoblast like- cells do not attach remain in suspension with a spherical shape
3. Fibroblast like- cells attached to an substrate appears elongated and bipolar

Types of cell culture

Cell culture is classified into three types:

1. Primary cell culture
   - Adherent cell culture
   - Suspension cell culture
2. secondary cell culture
3. Cell line
   -finite cell line
   - continuous cell line

Four stages of primary culture:

1. Obtained of the sample.
2. Isolation of the tissue .
3. Dissection and/or disaggregation .
4. Culture after seeding into vessel culture .

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 disaggregation 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, to attach to a flat surface in order to survive and proliferate with maximum efficiency .

Transformed cells on the other hand, 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 combination. Such as : Elastase & Dnase for type II alveolar cell isolation

collagenase with Dispase and collagenase with hyaluronidase . There are other, non mammalian enzymes, such as 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, because the former contain other proteases as contaminations, although the latter are generally less toxic and more specific in there action.Trypsin and pronase give the most complete disaggregation, but may damage the cells.Collagenase and dispase, on the other hand, give incomplete disaggregation, but are less harmful.

Requirements & conditions of Tissues:.

1. Fat and necrotic tissue is best removed during dissection .
2. The tissue should be chopped finely with sharp instruments 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 form 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.
6. Isolation of specific cell types will probably require selective media.
7. Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

Materials & Solutions :

- mouse.
- Dissecting tools .
- Petri dishes .

- Pipettes .
- Tips .
- Dissecting stage .
- Incubator .
- Culture flasks .
- Centrifuge tubes .
- Ethanol 70% .
- Phosphate Buffer Saline (PBS) sterile.
- DMEM or RBMI 1640 media .
- Cups .

Protocol

- (1) Transfer tissue to fresh, sterile PBS, 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 cubes.
- (4) Transfer by pipette to a sterile centrifuge tube .
- (5) Allow the pieces to settle.
- (6) Wash by resuspending the pieces in PBS, 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 1ml growth medium.
- (9) Cap the flask, and place it in an incubator 37C for 18 _ 24 h .
- (10) If the pieces have adhered, then the medium volume may be made up gradually over the next 3 _5 days to 10ml and then changed weekly.

- (11) When observed the growth in culture you must transfer the tissue into fresh culture vessel .
- (12) Subculture if necessary .