ANIMAL CELL AND TISSUE CULTURE MANUAL

For the course of
BT 0312 – ANIMAL CELL AND TISSUE CULTURE LABORATORY

Offered to
III YEAR B.TECH., BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOENGINEERING
SRM UNIVERSITY
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LABORATORY SAFETY GENERAL RULES AND REGULATIONS

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 exogenous 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 the 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 or apron while working in the laboratory to protect clothing from contamination or accidental discoloration by staining solutions.
4. Closed shoes should be worn at all times in the laboratory setting.
5. Never apply cosmetics or insert contact lenses in the laboratory.
6. Do not smoke, eat, or drink in the laboratory. These activities are absolutely prohibited.
7. 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.
8. Never remove media, equipment, or especially, **bacterial cultures from the laboratory.** Doing so is absolutely prohibited.

9. 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.
10. Report accidental cuts or burns to the instructor immediately.
11. 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.
12. Do not lick labels. Use only self-stick labels for the identification of experimental cultures.
13. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.

The specific precautions outlined below must be observed when handling body fluids of unknown origin due to the possible imminent transmission of the HIV and hepatitis B viruses in these test specimens.

1. Disposal gloves must be worn during the manipulation of these test materials.
2. Immediate hand washing is required if contact with any of these fluids occurs and also upon removal of the gloves.
3. Masks, safety goggles, and laboratory coast should be worn if an aerosol might be formed or splattering of these fluids is likely to occur.
4. Spilled body fluids should be decontaminated with a 1:10 dilution of household bleach, covered with paper toweling, and allowed to react for 10 minutes before removal.
5. Test specimens and supplies in contact with these fluids must be placed into a container of disinfectant prior to autoclaving.

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

LABORATORY PROTOCOL

Student preparation for laboratory sessions

The efficient performance of laboratory exercises mandates that you attend each session fully prepared to execute the required procedures. Read the assigned experimental protocols to effectively plan and organize the related activities. This will allow you to maximize use of laboratory time.

PREPARATION OF EXPERIMENTAL MATERIALS

Microscope Slides

Meticulously clean slides are essential for microscopic work. Commercially precleaned slides should be used for each microscopic slide preparation. However, wipe these slides with dry lens paper to remove dust and finger marks prior to their use.

Labeling of culture vessels

Generally, microbiological experiments require the use of a number of different test organisms and a variety of culture media. To ensure the successful completion of experiments, organize all experimental cultures and sterile media at the start of each experiment. Label culture vessels with non- water- soluble glassware markers and / or self - stick labels prior to their inoculation. The labeling on each of the experimental vessels should include the name of the test organisms, the name of the medium, the dilution of sample, if any, your name or initials, and the date. **Place labeling directly below the cap of the culture tube.** When labeling Petri dish cultures, only the name of the organism(s) should be written on the bottom of the plate, close to its periphery, to prevent obscuring observation of the results. The additional information for the identification of the culture should be written on the cover of the Petri dish.

Inoculation Procedures

Aseptic techniques for the transfer or isolation of microorganisms, using the necessary transfer instruments is described fully in the experiments in Part I of the manual. Technical skill will be acquired through repetitive practice.

Inoculating Loops and Needles

It is imperative that you incinerate the entire wire to ensure absolute sterilization. The shaft should also be briefly passed through the flame to remove any dust or possible contaminants. To avoid killing the cells and splattering the culture, cool the inoculating wire by tapping the inner surface of the culture tube or the Petri dish cover prior to obtaining the inoculum.

When performing an aseptic transfer of microorganisms, a minute amount of inoculum is required. If an agar culture is used, touch only a single area of growth with the inoculating wire to obtain the inoculum. **Never drag the loop or needle over the entire surface, and take care not to dig into the solid medium.** If a broth medium is used, first tap the bottom of the tube against the palm of your hand to suspend the microorganisms. **Caution:** Do not tap the culture vigorously as this may cause spills or excessive foaming of the culture, Which may denature the proteins in the medium.

Pipettes

Use only sterile, disposable pipettes or glass pipettes sterilized in a canister. The practice of **pipetting by mouth has been discontinued** to eliminate the possibility of auto-infection by the accidental imbibement of the culture or infectious body fluids. Instead, a mechanical pipetting device is to be used to obtain and deliver the material to be inoculated.

Incubation Procedure

Microorganisms exhibit a wide temperature range for growth. However for most used in this manual, optimum growth occurs at 37^0C over a period of 18 to 24 hours. Unless otherwise indicated in specific exercise, incubate all cultures under the conditions cited above. Place culture tubes in a rack for incubation. Petri dishes may be stacked; however, they **must always be incubated in an inverted position (top down)** to prevent water of condensation from dropping onto the surface of the culture medium. This resultant excess moisture may then serve as a vehicle for the spread of the micro-organisms on the surface of the culture medium, thereby producing confluent rather than discrete microbial growth.

PROCEDURE FOR RECORDING OBSERVATIONS AND RESULTS

The accurate accumulation of experimental data is essential for the critical interpretation of the observations upon which the final results will be based. To achieve this end, it is imperative that you complete all the preparatory readings that are necessary for your understanding of the basic principles underlying each experiment. Meticulously record all the observed data in the "Observations and Results" section of each experiment.

In the exercises that require drawings to illustrate microbial morphology, it will be advantageous to depict shapes, arrangements, and cellular structures enlarged to 5 to 10 times their actual microscopic size, as illustrated below. For this purpose a number 2 pencil is preferable. Stippling may be used to depict different aspects of cell structure, e.g., endospores or differences in staining density.

Review Questions

The review questions are designed to evaluate understanding of the principles and the interpretations of observations in each experiment. Completion of these questions will also serve to reinforce many of the concepts that are discussed in the lectures. The designated critical-thinking questions are designed to stimulate further refinement of cognitive skills.

Procedures For Termination of Laboratory Session

1. All equipment, supplies and chemical reagents are to be returned to their original locations.
2. All capped test-tube cultures and closed Petri dishes are to be neatly placed in a designated collection area in the laboratory for subsequent autoclaving.
3. Contaminated materials, such as swabs, disposable pipettes, and paper towels, are to be placed in a biohazard receptacle prior to autoclaving.
4. Hazardous biochemicals, such as potential carcinogens, are to be carefully placed into a sealed container and stored in a fume hood prior to their disposal according to the institutional policy.

Cleaning and preparation of cleaning solution

The need for cleaning

i) To remove the stains in the glassware.

ii) To remove the chemical residues.

iii) To remove the microbes partially by using the cleaning solution.

iv) To remove the impurities which were stick on the surfaces of the glass wares.

v) To remove the greasy areas by using mild detergents.

Preparation Of Cleaning Solution

AIM : To prepare the cleaning solution to clean the glass wares.

Requirements: Balance, Erylyn Meyer flask (1 L), measuring cylinder, spatula, butter paper, potassium dichromate, concentrated sulphuric acid, metal distilled water etc.

Composition for Cleaning Solution No:

DILUTE SOLUTION	**CONC.SOLUTION**	
Potassium dichromate	60 g	60 g.
Water	1 litre	300 ml.
Conc.sulphuric acid	60 ml	460 ml.

Procedure

About 800 ml of distilled water contained in a clean erylyn Meyer flask was dissolved with 60 g of potassium dichromate and mixed with 60 g of potassium dichromate and mixed with 200 ml of conc. H_2SO_4 and made upto a volume of 1 litre with distilled water. This solution is allowed to cool and used later.

This cleaning solution which can be used to oxidise any organic matter and will clean the glassware like test tubes, Petriplates, pipettes etc. The cleaning solution can be used until it terms into dark green colour solution.

Cleaning

The glassware are soaked in the cleaning solution overnight and washed with soap water and rewashed in running tap water and then with distilled water. Glassware were allowed to drain of water and dried in a hot air oven at 80^0C for 2 hrs for further use.

SAFETY IN LABORATORIES

General safety measures

Laboratory safety may appear at first sight to be rather a dull subject and the temptation may be to read this section only superficially or not at all. However, the view of the subject changes rapidly if you find yourself in the middle of a first or the victim of an accident and by this time ignorance can be dangerous or even fatal.

Laboratory safety equipment

Laboratories can be dangerous places in which to work and all users need to be aware of the potential hazards and to know what to do in case of emergency. When starting work in a new laboratory, it is important to become familiar with the layout of the room and the location of the safety equipment. The position of the emergency exits, first alarm and extinguishers should be known so that appropriate action can be taken in the event of fire. It is also important to know where the telephones are so that help can be summoned swiftly and to know the whereabouts of the first aid box so that rapid assistance can be given to an injured person. The main taps for gas and water and the switch for electricity should also be located so that these services can be turned off in an emergency.

The person incharge of class should of course point out where the safety equipment is located and also drawn attention to any specific hazards to be ground in a particular experiment.

Safety notices

Laboratory workers must also know the meaning of safety sings. Some of these are in plain English while others are in the form of pictograms. The sings have been standardized in Britain and Europe in terms of lettering, diagrams and colour so they can be rapidly identified; some examples of these are given in Fig.1.1.

Personal Protection

Goggles or safety spectacles Eyes are especially vulnerable to splashes from reagents and safety spectacles should always be worn when carrying out any procedures where there is a risk.

Gloves Heavy-duty gloves must be worn when handling corrosive substances such as strong acids or alkalis . The hazardous nature of these substances is obvious but the dangers inherent in skin contact with other chemicals are not always clear.

Lightweight disposable gloves should therefore be worn during weighing and handling of chemicals to avoid the risk of absorption through the skin.

Protection Clothing Laboratory coats are not status symbols but are meant to protect the wearer from chemical splashes and infection material. Cotton is a better material for a lab coat that nylon as it has a great absorptive capacity and is generally more resistant to chemical splashes.

The standard open-neck coat may be adequate for most chemical work but a high necked gown is more suitable for work with animals and potentially dangerous micro. Organisms.

Face masks these are not always necessary but need to be worn when there is a risk of dust from chemicals or an aerosol of micro-organisms.

Dangers to avoid

Poisoning often arises from the accidental transfer of a compound to the mouth and this risk can be greatly reduced by always keeping three simple rules in the laboratory.

1. No smoking
2. No eating and drinking
3. No mouth pipetting

Chemical hazards

All chemicals should be considered potentially dangerous and handled accordingly. Contact with skin and clothing should be avoided and even if a chemical is though to be harmless it should not be tasted or smelt.

Hazard warning symbols, which are black on an orange background, are present on reagent bottles to warn of specific dangers and must be heeded. Solutions of reagents placed out for classwork should also be marked by the technical staff and colored adhesive labels are available for this purpose.

Corrosive and irritant substances

A corrosive substance is one that destroys living tissue and the inherent dangers of strong acids or alkalis coming in contact with the skin are only too obvious.

An irritant on the other hand cause local inflammation but not destruction of the tissue and the dangers in this case are more subtle and not always appreciated. For example, occasional contact with the skin may suggest that the substance has no detectable effect. However, repeated exposure can suddenly give rise to irritation as in the case of some organic solvents.

Toxic Compounds

Compounds are graded as toxic or highly toxic depending on the dose required to kill 50% of a population of animals (LD_{50}). The inherent dangers of swallowing a toxic compound are obvious but the dangers of absorption through the skin or inhalation are not always appreciated.

Some compound take a long time before their toxicity becomes evident and this is particularly true fir carcinogens and teratogens. Some common biochemical reagents show this long-term toxicity, ninhydrin for example is carcinogenic and thyroxin is teratogenic, If possible a substitute should always be used but if none is available then extra care must be taken when using these substances.

Flammability hazards

Flammable substances are those with a flash point and all naked flames in the laboratory should be extinguished when handling them and not only those in the immediate vicinity of the substances, Sparks from electrical equipment are less obvious then a Bunsen burner but can be just as dangerous. For this reason, organic solvents must not be stored in the refrigerator.

Oxidizing substances may not be flammed themselves but may cause a fire when brought into contact with combustible material.

The best precaution if such compounds need to be used, is to have only the minimum amount required on the bench and to keep the main bulk in steel cabinets well away from the work area.

Explosives

Explosives as such are not handled in the normal biochemical laboratory but some general laboratory or reagents such as picric acid are explosive and must be handled with extreme caution.

As with flammable compounds, only small quantities of the compound should be used in the work area and preferably behind a protective screen. Explosion can also arise from the mixture of two compounds which in themselves are harmless and an awareness of this is necessary to avoid a laboratory disaster.

INTRODUCTION FOR 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 every thing you use with your Reg. number, date and name of the reagent, buffer or medium.

Handling Methods

- Use sterile glassware and pipettes.
- When you open a glass bottle, before and after use flame the mouth in a flame.
- Flame glass pipettes.
- Use rubber bulbs to control pipettes. DO NOT MOUTH PIPETTE. This is to protect both you and the cells from contamination
- Prewarm medium and serum to 37ºC in water bath.
- Wipe bottles with filter paper and transfer to the tissue culture hood (Laminar Air Flow).
- Open bottles, flame tops, replace caps loosely but so they won't fall off.
- Transfer, desired quantity of serum to the medium, or mix in separate (sterile) container.
- Using sterile but NOT plugged, Pasteur pipette, aspirate the medium. Use a separate pipette for different cells.
- Using sterile pipette, transfer desired amount of medium and serum to the dish. Do not reuse the pipette.
- Return dishes to incubator without shaking to avoid spill-over of medium.
- Reflame tops of bottles and close tightly using aluminium foil. Close pipette cans.
- Work only in designated tissue culture area and Wipe surfaces with ethanol before starting.
- Work cleanly, mopping spills immediately.
- Discard used medium, and especially contaminated plates/TC bottles carefully. Otherwise, they serve as sources of contamination.
- Label bottles and dishes with Reg. No., cell type, date etc. When you open a new bottle of medium or serum, write the date on it and indicate how much has been removed.
- If you add anything to medium, indicate this on the bottle. If there is only a small amount left in a bottle, discard it.
- After you have finished, remove your belongings; discard properly. Eventually, wipe the working surface with ethanol.
- A sterile hood is available for your use. Keep UV, lamp on for at least 10 mins before use. Turn off the UV lamp while you work.

Sterile glassware
Sterile pipettes } Autoclaved

Sterile medium
Sterile serum } Millipore – filtered (0.45u)

EXPERIMENT NO: 1

DATE:

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 by or growth of undesirable microorganisms 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, they act in many different ways and each has kits own limits of applications.

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.

TYPES OF STERLISATION TECHNIQUES

AUTOCLAVE

The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. The autoclave is an essential unit of equipment in every microbiology or cell culture laboratory. Many media, solutions, discarded cultures, and contaminated materials are routinely sterilized with this apparatus. Generally, but not always, the autoclave is operated at a pressure of approximately 15lb/in^2 (121^{0}C). The time of operation to achieve sterility depends on the nature of the material being sterilized, the type of the container, and the volume. For example, 1000test tubes containing 10ml each of a liquid medium can be sterilized in 10 to 15 min at 121^{0}C, 10 litres of the same medium contained in a single container would require 1hr or more at the same temperature to ensure sterilization..

BOILING WATER

Contaminated materials exposed to boiling water cannot be sterilized with certainty. It is true that all vegetative cells will be destroyed within minutes by exposure to boiling water, but some bacterial spores can withstand this condition for many hours. The practice of exposing instruments for short periods of time in boiling water is more likely to bring about disinfection(destruction of vegetative cells of disease producing microorganisms) rather than sterilization. Boiling water cannot be used in the laboratory as a method of sterilization..

DRY HEAT OR HOT AIR OVEN

Dry hear or hot air sterilization is recommended where it is either undesirable or unlikely that steam under pressure will make direct and complete contact with the materials to be sterilized. This is true of certain items of laboratory glass wares, such as Petri dishes and pipettes, as well as oils, powders and similar substances. The apparatus employed for this t7ype of sterilization may be a special electric or gas oven or even the kitchen dry oven. For laboratory glassware, a 2h exposure to a temperature of 160^0C is sufficient for sterilization.

ULTRAVIOLET LIGHT

The ultraviolet portion of spectrum includes all radiation from 150 to 3900 A. Wavelengths around 2650A have the highest bactericidal efficiency. Although the radiant energy of sunlight partly composed of ultraviolet light, most of the shorter wavelengths of this type are filtered out by the earth's atmosphere. Consequently, the ultraviolet radiation at the surface of the earth is restricted to the span from about 2670A to 3900A. From this, we may conclude that sunlight under certain conditions, has microbicidal capacity, but to a limited degree. An important practical consideration in using this means of destroying microorganisms is that ultraviolet light has very little ability to penetrate matter. Even as thin layer of glass filters off a large percentage of the light. Thus, only the microorganisms on the surface of an object where they are exposed directly to the ultraviolet light are susceptible to destruction.

FILTRATION

For many years a variety of filters have been available to the microbiologist which can remove microorganisms from liquids or gases. These filters are made of different materials – an asbestos pad in the Seitz filter, diatomaceous earth in the Berkeloid filter, porcelain in the Chamberland-Pasteur filter and sintered glass disks in other filters.The mean pore diameter in these bacteriological filters ranges from approximately one to several microfilters; most filters are available in several grades based on the average size of the pores. However, it should be understand that these filters do not act as mere mechanical sieves; porosity alone is not the only factor preventing the passage of organisms. Other factors, such as the electric charge of the filter, the electric charge carried by the organisms, and the nature of the fluid being filtered, can influence the efficiency of filtration.

In recent years a new type of filter termed the membrane or molecular filter has been developed whose pores are of a uniform and specific predetermined size. Membrane or molecular filters are composed of biologically inert cellulose esters. They are prepared as circular membranes of about 150µm thickness and contain million of microscopic pores of very uniform diameter. Filters of this type can be produced with known porosities ranging from approximately 0.01 to 10µm. Membrane filters are used extensively in the laboratory and in industry to sterilize fluid materials. They have been adapted to microbiological procedures for the identification and enumeration of microorganisms from water samples and other materials. It is customary to form the fluid through the filter by applying a negative pressure to the filter flask by use of a vacuum or water pump or to impose a positive pressure above the fluid in the filter chamber, thus forcing it through. Upon completion of filtration, precautions must be taken to prevent contamination of the filtered material.

EXPERIMENT NO: 2

DATE :

PREPARATION OF MEDIA

AIM : To prepare desired medium for the given Animal cell culture.

PRINCIPLE

All the Animal cells can be grown in a liquid culture medium consisting of a mixture of vitamins, salts, glucose, amino acids and growth factors. Moreover, Calf serum is an easily available source of growth and attachment factors. Antibiotics are added to prevent the growth of bacteria. Under these conditions cells will grow at physiological pH (7.4) and at body temperature (37ºC) to form a monolayer on the culture vessels.

MATERIALS REQUIRED

Medium

Adult bovine serum

Membrane filter (Millipore 0.45μ)

Sterilize

Double distilled water 1000 ml

1 litre measuring cylinder

100 ml measuring cylinder

1 litre filtration flask

Medium storage bottles

Other Glasswares

Method:

Sterilize the laminar air flow by UV irradiation for 45 minutes before using it.

1. Take 500ml of sterile double distilled water in a 1000 ml measuring cylinder.
2. Transfer the contents of the powdered medium into 1 litre measuring cylinder add 3.7 gms of $NaHCO_3$ in the absence of CO_2 incubator.
3. Mix thoroughly to dissolve the powdered medium, and add penicillin /streptomycin/gentamycin.

4. Fill the cylinder with1 litre double distilled water mix and transfer to sterile 2 litre flask and mix. Pinkish red color of the medium indicates normal pH range.
5. Assemble the filter sterilization set-up and carry out the filtration under negative pressure.
6. Prepare 400 ml of medium containing 10% Adult bovine serum using 100 ml measuring cylinder and store in a 500 ml sera lab bottle.
7. Transfer the remaining medium without serum into big glass bottles.
8. Store the medium in refrigerator, dispose the used membrane and immerse the used glassware in water for washing.
9. Different types of medium is used for various kind of Experiments.
10. The components of different types of medium is given in the following tables.

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	BME
Inorganic salts (mg/l)	
	6800.00
NaCl	400.00
KCl	200.00
CaCl2	--
CaCl2.2H2O	--
MgCl2.6H2O	200.00
MgSO4.7H2O	150.00
NaH2PO4.2H2O	2200.00
NaHCO3	
L-Amino acids (mg/l)	
	--
Alanine	21.00
Arginine--HCl	--
Asparagine.H2O	--
Aspartic acid	--
Cysteine-HCl.H2O	12.01
Cysteine	--
Cysteine-diNa	--
Glutamic acid	10.50
Histidine-HCl.H2O	26.23
Isoleucine	26.23
Leucine	36.52
Lysine-HCl	7.46
Methionine	16.51
Phenylalanine	--
Proline	--
Serine	23.82
Threonine	4.02
Tryptophan	18.11
Tyrosine	--
Tyrosine-diNa	23.43
Valine	

Trace elements (mg/l)	
Fe(NO3)3.9H2O	--
Vitamins/cofactors(mg/l)	
	--
Ascorbic acid	1.00
Biotin	1.00
Choline-Cl	1.00
Folic acid	--
Inositol	1.00
Nicotinamide	1.00
Pantothenate-Ca	1.00
Pyridoxal-HCl	0.10
Riboflavin	1.00
Thiamine-HCl	--
Vitamin B112	
Nucleosides and ribonucleosides(mg/l)	
	--
Adenosine	--
Cytidine	--
2-Deoxyadenosine	--
2-Deoxycytidine	--
2-Deoxyguanosine	--
Guanosine	--
Thymidine	
Uridine	
Other components(mg/l)	10.00
Phenol red	1000.00
Glucose	--
Pyruvate-Na	5
CO2(gas phase)(%)	

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	EMEM
Inorganic salts(mg/l)	
NaCl	6800.00
KCl	400.00
CaCl2	200.00
CaCl2.2H2O	--
MgCl2.6H2O	--
MgSO4.7H2O	200.00
NaH2PO4-2H2O	150.00
NaHCO3	2200.00
L-Amino acids (mg/l)	
Alanine	--
Arginine	126.40
Asparagine-HCl	--
Aspartic acid	--
Cysteine-HCl.H2O	--
Cysteine	24.02
Cysteine-diNa	--
Glutamic acid	--
Glutamine	292.30
Glycine	--
Histidine-HCl.H2O	41.90
Isoleucine	52.50
Leucine	52.50
Lysine-HCl	73.06
Methionine	14.90
Phenylalanine	33.02
Proline	--
Serine	--
Threonine	47.64
Tryptophan	10 20
Tyrosine	36.22

Tyrosine-diNa	--
Valine	46.90
Trace elements (mg/l)	
Fe(NO3)3.9H2O	--
Vitamins/cofactors (mg/l)	
Ascorbic acid	--
Biotin	--
Choline.Cl	1.00
Folic acid	1.00
Inositol	2.00
Nicotinamide	1.00
Pantothenate-Ca	1.00
Pyridoxal-HCl	1.00
Riboflavin	0.10
Thiamine-HCl	1.00
Vitamine B12	--
Nucleosides and ribonucleosides(mg/l)	
Adenosine	--
Cytidine	--
2-Deoxyadenine	--
2-Deoxycytidine	--
2-Deoxyguanosine	--
Guanosine	--
Thymidine	--
Uridine	--
Other components (mg/l)	
Phenol red	10.00
Glucose	1000.00
Pyruvate-Na	--
CO2 (gas phase)(%)	5

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	AMEM
Inorganic salts(mg/l)	
NaCl	6800.00
KCl	400.00
CaCl2	--
CaCl2.2H2O	264.90
MgCl2.6H2O	--
MgSO4.7H2O	200.00
NaH2PO4-2H2O	158.30
NaHCO3	2000.00
L-Amino acids (mg/l)	
Alanine	25.00
Arginine	126.40
Asparagine-HCl	50.00
Aspartic acid	30.00
Cysteine-HCl.H2O	100.00
Cysteine	24.02
Cysteine-diNa	--
Glutamic acid	75.00
Glutamine	292.00
Glycine	50.00
Histidine-HCl.H2O	41.93
Isoleucine	52.00
Leucine	52.00
Lysine-HCl	73.06
Methionine	15.00
Phenylalanine	33.00
Proline	40.00
Serine	25.00
Threonine	48.00
Tryptophan	10 00
Tyrosine	36.00

Tyrosine-diNa	--
Valine	46.86
Trace elements (mg/l)	
Fe(NO3)3.9H2O	--
Vitamins/cofactors (mg/l)	
Ascorbic acid	50.00
Biotin	0.10
Choline.Cl	1.00
Folic acid	1.00
Inositol	2.00
Nicotinamide	1.00
Pantothenate-Ca	1.00
Pyridoxal-HCl	1.00
Riboflavin	0.10
Thiamine-HCl	1.00
Vitamine B12	1.36
Nucleosides and ribonucleosides(mg/l)	
Adenosine	40.00
Cytidine	10.00
2-Deoxyadenine	10.00
2-Deoxycytidine	10.00
2-Deoxyguanosine	10.00
Guanosine	10.00
Thymidine	10.00
Uridine	10.00
Other components (mg/l)	
	10.00
Phenol red	1000.00
Glucose	110.00
Pyruvate-Na	
CO2 (gas phase)(%)	

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	DMEM
Inorganic salts(mg/l)	
NaCl	6400.00
KCl	400.00
CaCl2	--
CaCl2.2H2O	264.90
MgCl2.6H2O	--
MgSO4.7H2O	200.00
NaH2PO4-2H2O	140.00
NaHCO3	3700.00
L-Amino acids (mg/l)	
Alanine	--
Arginine	84.00
Asparagine-HCl	--
Aspartic acid	--
Cysteine-HCl.H2O	--
Cysteine	--
Cysteine-diNa	56.78
Glutamic acid	--
Glutamine	584.60
Glycine	30.00
Histidine-HCl.H2O	42.00
Isoleucine	104.80
Leucine	104.80
Lysine-HCl	146.20
Methionine	30.00
Phenylalanine	66.00
Proline	--
Serine	--
Threonine	95.20
Tryptophan	16.00
Tyrosine	--

Tyrosine-diNa	89.46
Valine	93.60
Trace elements (mg/l)	
Fe(NO3)3.9H2O	0.10
Vitamins/cofactors (mg/l)	
Ascorbic acid	--
Biotin	--
Choline.Cl	4.00
Folic acid	4.00
Inositol	7.00
Nicotinamide	4.00
Pantothenate-Ca	4.00
Pyridoxal-HCl	4.00
Riboflavin	0.40
Thiamine-HCl	4.00
Vitamine B12	--
Nucleosides and ribonucleosides(mg/l)	
Adenosine	
Cytidine	
2-Deoxyadenine	. --
2-Deoxycytidine	--
2-Deoxyguanosine	--
Guanosine	--
Thymidine	--
Uridine	--
	--
Other components (mg/l)	--
Phenol red	
Glucose	10.00
Pyruvate-Na	4500.00
CO2 (gas phase)(%)	110.00
	10

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	GMEM
Inorganic salts(mg/l)	
NaCl	6400.00
KCl	400.00
CaCl2	--
CaCl2.2H2O	264.90
MgCl2.6H2O	--
MgSO4.7H2O	200.00
NaH2PO4-2H2O	140.00
NaHCO3	2750.00
L-Amino acids (mg/l)	
Alanine	--
Arginine	42.12
Asparagine-HCl	--
Aspartic acid	--
Cysteine-HCl.H2O	--
Cysteine	--
Cysteine-diNa	28.42
Glutamic acid	--
Glutamine	584.60
Glycine	--
Histidine-HCl.H2O	21.00
Isoleucine	52.46
Leucine	52.46
Lysine-HCl	73.06
Methionine	14.92
Phenylalanine	33.02
Proline	--
Serine	--
Threonine	47.64

Tryptophan	8.16
Tyrosine	--
Tyrosine-diNa	45.02
Valine	46.86
Trace elements (mg/l)	
Fe(NO3)3.9H2O	0.10
Vitamins/cofactors (mg/l)	
Ascorbic acid	--
Biotin	--
Choline.Cl	2.00
Folic acid	2.00
Inositol	4.00
Nicotinamide	2.00
Pantothenate-Ca	2.00
Pyridoxal-HCl	2.00
Riboflavin	0.20
Thiamine-HCl	2.00
Vitamine B12	--
Nucleosides and ribonucleosides(mg/l)	
Adenosine	
Cytidine	. --
2-Deoxyadenine	--
2-Deoxycytidine	--
2-Deoxyguanosine	--
Guanosine	--
Thymidine	--
Uridine	--
Other components (mg/l)	--
Phenol red	
Glucose	
Pyruvate-Na	
CO2 (gas phase)(%)	10.00
	4500.00

EAGLE'S MEDIUM AND DERIVATIVES (mg/l)

Components	JMEM
Inorganic salts(mg/l)	
NaCl	6500.00
KCl	400.00
CaCl2	--
CaCl2.2H2O	--
MgCl2.6H2O	200.00
MgSO4.7H2O	--
NaH2PO4-2H2O	1500.00
NaHCO3	1500.00
L-Amino acids (mg/l)	
Alanine	--
Arginine	126.40
Asparagine-HCl	--
Aspartic acid	--
Cysteine-HCl.H2O	--
Cysteine	24.00
Cysteine-diNa	--
Glutamic acid	--
Glutamine	294.00
Glycine	--
Histidine-HCl.H2O	42.00
Isoleucine	52.00
Leucine	52.00
Lysine-HCl	73.06
Methionine	15.00
Phenylalanine	32.00
Proline	--
Serine	--
Threonine	48.00

Tryptophan	10.00
Tyrosine	36.00
Tyrosine-diNa	--
Valine	46.00
Trace elements (mg/l)	
Fe(NO3)3.9H2O	
	0.10

EXPERIMENT NO: 3

DATE :

PREPARATION OF SERA

Aim : To prepare serum from the given blood sample.

Principle

Serum is a natural biological fluid are rich in various components to support cell proliferation. The most commonly used sera are Calf serum, Fetal Calf serum and Horse serum. Approximately 5-20% of serum is mostly used for supplementary serial media. The salient features of the serum constituents are

Serum used in promoting cell attachment and growth of the cell.

Serum proteins increase the viscosity of the culture media.

Serum growth factors will stimulate the proliferation of cells in the culture.

Serum hormones will promote cell attachment, glucose uptake and cell proliferation.

Materials Required

Beaker

Conical flask

Centrifuge tubes

Water bath

Micro centrifuge

Variable pipette

Serum bottle

Membrane filter system

Goat blood

Method

All steps should be carried out on ice unless otherwise stated.

1. Collect 1 litre of goat blood aseptically from slaughter house.
2. Keep at room temp for 30 min for clotting.
3. Keep at 4ºC for 2-3 hours for the clot to shrink.
4. Transfer the clear serum to 500 ml flask.
5. Distribute into 50 ml tubes and centrifuge at 2000 rpm for 10 mins at 4ºC.
6. Collect the supernatant into 500 ml flask and distribute into six 30 ml. corex tubes. Centrifuge at 10,000 rpm for 10 min at 4ºC.
7. Collect the supernatant into 500 ml flask and transfer into laminar flow hood.

8. Inactivate the complement components by keeping the serum bottle in a water bath at 56ºC for 30 min.
9. Allow it to come to room temp and store it at -20ºC for further use.
10. Sterilize the serum by passing through Millipore membrane and transfer into sterile serum bottles and cover with tin foil.

EXPERIMENT NO:4

DATE :

PRIMARY CELL CULTURE

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

Introduction:

Development of techniques for the *in vitro* culture of animal cells has proven valuable for the study of structure and function of cells under controlled conditions. Further, cultured cells find important applications in vaccine production, hybridoma production and in chromosome karyotyping. Almost any tissue can be cultured, if it is appropriately dispersed, though high rates of success in cell culture is often recorded with embryonic and tumor tissues rather than normal adult tissues.

Cultures started fresh from tissues are called primary cultures. A method for the propagation of primary cultures of mouse embryo cells is given below which can be adopted for the culture of other embryonic tissues derived from different species. Often primary and secondary cultures derived from normal tissue have finite life span similar to their *in vitro* life. However, some cells out of a large population are secondary cultures by pass this definite life span and get immortalized with a capacity to divide indefinitely and these are called cell lines. Many cancer cells have a capability to divide indefinitely in culture. Normal cells transformed by viruses and chemical carcinogens also become continues cell lines.

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.

Materials Required:

13 – 14 days pregnant mouse / 8-10 days old embryonic eggs.

100 ml beaker – 1

2 pairs of scissors

A pair of bent scissors

2 big forceps

Petriplates – 2 pairs

100 ml conical flasks

Small funnel covered with

cheese cloth – 1

10 ml testubes cotton plugged – 4

Trypsinization flask – 1

Growth medium (M.199 with 10% ox serum)

Calcium, Magnesium free – phosphate buffered saline (PBS).

METHODS

MOUSE EMBRYO FIBROBLASTS

Mouse embryos of age 13-15 days are needed for culture. To get these, Swiss mice were kept for mating and the gestation period was times by designating the day of finding the genital plug as the first day of development.

1. Sacrifice the pregnant mouse by cervical dislocation. Place the animal in supine position.
2. Swab the abdomen with 70% ethanol and cut open along the midventral line.
3. Remove uterine horns and transfer into a beaker containing PBS. Transfer into flow hood immediately.
4. Transfer the uterine horns into petriplates containing PBS inside the flowhood and cut open the uterine horns to remove embryos.
5. Wash the embryos with PBS and remove head, visceral organs and appendages.
6. Transfer the remains of the embryos to another petriplate containing small amount of PBS and mince thoroughly with a pair of bent scissors.
7. Transfer the minced tissue to the trypsinization flask containing 40 ml of 0.25% trypsin in PBS.
8. Stir the contents at 37ºC for 30-60 mins.
9. At the end of the above period add 5ml of medium containing serum and stir the contents for 2 more minutes to inactivate the action of trypsin.
10. Filter the cell suspension through sterile cheese cloth and collect the filtrate into a 100 ml conical flask.
11. Centrifuge the filtrate at ~ 1000 rpm for 10mins.
12. Pour out the supernatant and resuspend the pellet in 5 ml of medium. Distribute equally to all the 120 cm^2 culture bottles and incubate at 37ºC.

Chick Embryo Fibroblasts

The procedure for culturing CHF is the same as for MEF. Briefly remove embryos from 8-10 day old embryos, break the shell with the help of foreceps and transfer the embryo into petriplates containing PBS and follow steps 5 to 12 of mouse embryo fibroblasts culture (see above). Although the chick embryo cells will grow in the same medium, they will grow better if 1% chicken serum is also added. Instead of calf serum you can substitute Millipore-filtered goat serum (10%) for the culturing of above cells.

EXPERIMENT NO : 5

DATE :

PREPARATION OF ESTABLISHED CELL LINES

Aim : To develope secondary growth or established cells from primary culture by repeated subculture.

Principle

Cells which originate by subculture of a primary culture are called cell lines. Frequently primary cell lines go on dividing at quite a high rate for a long time and can be passaged repeatedly. At times few cells may become altered in such a way that they acquire a different morphology, grow faster and multiply. These cells can be cultured for a long time and can even be subcultured indefinetly in vitro. Such cell lines are called established cell lines.

Materials required

Monolayer cells(chick embryo)

Beaker

TC bottles

Trypsinization flask

Growth medium

Phosphate buffer saline

Pasteur pipette

Trypsin

Methods

1. Take Tc bottle containing a fully formed monolayer of cells.
2. Discard the old medium and wash the monolayer thrice with PBS.
3. Add 5-6 drops of 0.25% trypsin and allow the drops to spread over the entire monolayer.
4. Wait for a minute and shake the bottle vigorously to facilitate the cells to come off the substratum.
5. Once the cells start coming off then add 5ml of medium containing serum. Flush with Pasteur pipette to dislodge the cells adhering to the glass surface. Divide the cells suspension into two bottles and incubate at 37ºC.
6. Replace the fresh medium for every three days to remove the dead cells along with the old medium.
7. The above steps should be repeated for 70 times to yield established cell lines.

Result

During repeated subculturing cell lines can undergo extensive changes in their properties i.e. the cells may grow in clumps rather than in monolayer, orientation of cells may be irregular. Such cell lines are said to be transformed and most commonly they are neoplastic. The established cell lines are also known to have unusual number of chromosomes.

EXPERIMENT NO : 6

DATE :

CELL COUNTING AND VIABILITY

AIM : To ensure the population of cells required for the culture works by cell counting method and its viability by vital staining methods

Introduction

Haemocytometer (also known as hemocytometer) is a glass slide with two counting chambers etched in a surface area of 9mm square. Each chamber is divided into nine 1.0mm square. It has raised sides which keep the cover slip 0.1mm above the chamber floor so that the total volume of each square becomes 0.0001ml(1.0mm x 0.1mm or 0.1mm² or 10^2 cm³, L x W x H).

Principle

Staining of cells identifies viable cells. Stains generally used are Trypan Blue, Erythrosin B and Nigrosin. Nuclei of damaged or dead cells take up the stain whereas the viable cells do not do so.

Requirements

Cell suspension

Spirit lamp

Hemocytometer

Microscope

Micropipette

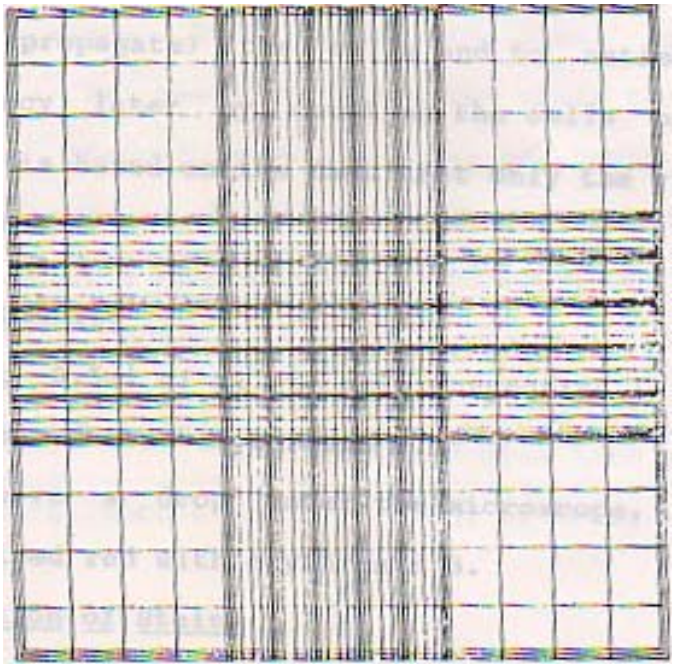
PBS

Tryphan Blue 0.4%

Methodology

1. Take the Hemocytometer and place it on the flat surface of the work bench. Place the cover slip on the counting chamber.
2. Mix 20µl cells that have been well mixed prior to sampling with an equal volume of tryphan blue.
3. Apply to a hemocytometer by pipetting from the edge of the cover slip and permitting diffusion by capillary action.
4. Make sure that there is no air bubble and there is no overfilling beyond the ruled area.
5. Leave the counting chamber on the bench for 2-3 minutes to allow the cells to settle.
6. Place the counting chamber on the stage of the microscope between the clips to the hold slide so that the counting chamber can be moved (if the microscope is provided with a moving stage).
7. Switch to low power (10x) objective, adjust the light (less light needed, hence close the aperture or lower the condenser) and focus on the wall of the counting chamber.
8. Then slowly move the stage towards the middle of the slide until the ruling area visible, sharpen the focus and locate the large square in the centre.
9. Locate the large square in the centre with 25 small squares. Place in the middle of the field of vision and examine the distribution of viable cells on the entire area. It must be uniform orelse refill the chamber with cell suspension.
10. Carefully switch to high power objective (40 x) and move the chamber so that the smaller upper left corner square (with 16 smaller squares) is completely in the field of vision.

11. Count the number of unstained cells seen on the small square (0.2x0.2=0.04sq mm) of the upper left corner which is divided into 16 smaller squares to facilitate counting.
12. Repeat the counting with three other corner squares.
13. Make a total of all the cells counted in 4 squares. Repeat the same on the other side of the chamber and make an average of the two chambers.



Result

The concentration of cells in the original suspension in cells/ml =

No. of cells counted

---------- $X10^4$ x 2

No. of Grids counted

----------/ml

EXPERIMENT NO : 7

DATE :

STAINING OF ANIMAL CELLS

Aim : To ensure the differentiation of live cells from dead cells by giemsa stain method.

Introduction

A number of vital staining procedures have been developed to have quick quantitation of living cells. Quite often, when tissues are dispersed to obtain cells, a substantial proportion of cells are killed. So before proceeding further, it is necessary to know the percentage of living cells. Some staining procedures depend on the metabolic activity of cells (e.g. staining with methylene blue) while others using trypan blue or erythrosine B depend on the membrane transport properties of cells. Living cells exclude (do not take up) these stains. These vital staining procedures are very arbitrary and should be used with reservations. The best way to determine viability is to plate (propagate) the cells and to estimate the plating efficiency later by counting the cells or nuclei. This method is based on the fact that only the viable cells will replicate.

Vital Staining

1. Keep 0.5 ml of dilute cell suspension in a tube and add 0.1 ml of 0.4% erythrosin B or Trypan Blue.
2. Observe a drop under the microscope. Dead cells are stained red with erythrosin B or Trypan Blue.

Preparation of Erythrosin B Stain

Dissolve 0.4 g of Erythrosin B in 100ml sterile PBS.

Giemsa staining

Preparation of 10X Giemsa Stain

1. Add 20ml glycerol to 0.3g Giemsa powder and keep in a water bath at 56ºC, 2 hours.
2. Then add 20ml of methanol and mix well. Leave it for 7 days at RT then filter.

Staining Method

1. Rinse Monolayer with PBS
2. Fix with 10ml of 3:1 Methanol : acetic acid or ethanol
3. Drain fixer and air dry
4. Dilute Giesma 1 to 10 (10ml + 90ml H_2O) and use 20 ml of diluted stain for staining for 30 min.
5. Discard stain, rinse several times with water, finally with tap water, air dry and look under microscope.

Observation

The viable cells appears unstained and the dead cells looks red colour, this results because of manual disturbance (chopping) or chemical disturbance (tripsinisation). The cell membrane gets damage by disaggregation so the stain penetrates into the damaged cells not in normal cell.

EXPERIMENT NO : 8

DATE :

PRESERVATION 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 a discernible 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 in the freezing and storage medium and,

4) Storage at temperatures below -70ºC.

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 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.

Procedure:

1. Trypsinize the cells and add 5 ml of 20% FCS containing medium.
2. Slowly add 0.5 ml of glycerol or DMSO to a final concentration of 10%.
3. Mix them thoroughly and transfer 1ml into each CRYO tube.
4. Store them in a sterile beaker at 4ºC overnight.
5. After 8-10 hours transfer them to -70ºC.
6. Eventually transfer to -196ºC .

To recover the cells from the freezer, thaw the cells rapidly at 37ºC and plate them in fresh 20% FCS containing medium in small 25 cm^2 flask and incubate them at 37ºC. Make a viable cell count before plating into the flask and calculate the percentage survival of cells.

EXPERIMENT NO: 9

DATE :

CULTURE OF VIRUS IN CHICK EMBRYO

Aim: To adapt and propagate New castle disease virus in chicken embryo.

Principle

Virus is an obligate endoparasite which can grow inside the host. It is effectively grown in embryonated egg i.e. viral suspension is inoculated into the egg allantoic membrane, where it infects the embryo by replication or multiplication. The infected embryo forms lesion on it.

Materials required

Eighth day embyonated egg

Iodine-alchol disinfectant

Syringe

Scotch tape

Incubator

NDV suspension culture

Method

1. Surface sterilize an embryonated egg**.**
2. Locate the position of air sac by holding the long axis of egg horizontally in front of a light source and mark the position.
3. Sterilize the needle by dipping in alcohol and then flaming it.
4. Use this needle to make a small hole or puncture the shell over the air sac. The membrane at base should not be punctured.
5. Inject 0.2ml of dilute viral suspension into the allantoic cavity. Seal the hole in the shell with scotch tape.
6. Maintain an uninoculated control by injecting 0.2ml of sterile saline and incubate at 37ºC in incubator.

Observation

Examine the egg periodically in front of light source for embryo death (usually takes 3-4 days). This is indicated by cessation of movement and disappearance of veins from egg shell. Once embryo death has been confirmed then crack the shell and collect the contents in a Petridish. Repeat the procedure with control egg. Compare the two embryos the infected embryo will have lesions on it. Virus can be separated from the infected tissue and purified.

EXPERIMENT NO : 10

DATE :

ADAPTATION OF VIRUS IN ANIMAL (*IN VITRO*) CELL CULTURE

Aim : Preparation of Suitable Cell Culture for the Adaptation of Animal virus and to study its Cytopathic effects.

Introduction

After deciding on the most suitable type of cells to be used, the required numbers of cultures are prepared in tubes or bottles with appropriate concentration of cells to form a uniform but not too heavy sheet of cells. The amount of medium to be used is related to the type of cells and main aim is to maintain the cells in the best possible conditions during the entire period of experiment. For this purpose, it is generally best to use a synthetic medium containing only small amount of added serum or other biological supplements which is well-buffered and will not therefore, require to be changed too often during the experiment.

Principle

Many Animal virus that replicate in the susceptible cells in cultures are capable of providing morphological changes that are visible either stained or unstained with inverted microscope or ordinary light microscope. These changes in the cells after viral infection is known as cytopathic [CPE]

Procedure

1. When the cell sheets have developed sufficiently, the exhauded tissue culture fluid is aseptically removed from each tube or bottle.
2. The monolayers are to be washed 2-3 times with warm PBS to remove the proteins and the dead cells.
3. The virus materials to be inoculated may be from another culture or from pathological material. The virus material is freed contamination by centrifugation, treatment with antibiotics or by filtration using a Swinney filter.
4. The cell monolayers are infected with virus material at the rate of 0.1 ml for milk dilution or prescription bottles, 1 ml for Roux flasks and 0.01 ml for Leighton tubes. The bottles or tubes are fitted in such a way that all the cells in monolayers are exposed to the virus material.
5. The monolayers are incubated at 37^0 C for 1 hour which facilitates adsorption of the virus into the cells.
6. Then the inoculum is discarded and maintenance medium is added and incubated at 37^0 C.
7. After infection at 24 hours intervals, the monolayers are examined for CPE.

Cytopathic effects due to virus activities are manifested in different ways and they are characteristic for each type of virus.

Observation

1. Complete destruction of the cell sheet (eg. Foot and Mouth disease virus)
2. The formation of multinucleated cells which are called “giant cells” and the dissolution of cell membranes with the fusion of cell cytoplasm to form “Syncytia” (Polykaryons) eg. Myxo viruses and herpes viruses.
3. The presence of intracytoplasmic or intranuclear inclusions (e.g. Pox viruses and Adeno viruses.)
4. Transformation of the cells as shown by their altered morphology and piled up massed of cells due to loss of contact inhibition (e.g. Papova viruses.)

5. Other changes in the appearance of the cytoplasm including increased granularity and distortion or fragmentation of the nucleus (e.g. Myxoma and some other Pox virus infections.)

EXPERIMENT NO : 11

DATE :

DPPH RADICAL SCAVENGING ASSAY

AIM

To evaluate the radical scavenging activity of the tissue hydrolysate against the DPPH free radical.

PRINCIPLE

DPPH (2, 2-Diphenyl-1-Picryl Hydrazyl) is relatively stable free radical. The bleaching rate of DPPH is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or radical species its absorption decreases. A lower absorbance of the reaction mixture would indicate higher free radical scavenging activity.

PROCEDURE I (Spectrometric method)

1. Tissue hydrolysate (1 ml) was added to a methanolic solution of DPPH radical (75 μmol L−1, 4mL).
2. The mixture was shaken vigorously and left in the dark at room temperature for 60 min, after which the absorbance was measured at 517 nm.
3. The DPPH-scavenging effect (%) was calculated as [(OD517 control −OD517 sample)/OD517 control] × 100, where OD is optical density.
4. The controls used were α-tocopherol and BHT.

CONCLUSION

The proton radical-scavenging action is known as an important mechanism of antioxidation. From this study the radical scavenging activity of the sample antioxidant could be observed. The DPPH assay is one of the examinations which will help determine the scavenging effect of the sample antioxidant against the free radicals and reactive oxygen species (ROS) which lead to various ailments.

ANIMAL HANDLING AND CARE

INTRODUCTION

Animals which are used for experimental purpose in biomedical veterinary and other research purposes are considered as laboratory animals. These animals either bred in captivity subsequent to demonstration or produced from its environment as wild relates to their fitness for specific investigation.

ANIMAL MODELS

Animals like Rats and Mouse have better adaptability by their nature as Omnivores in habit, robust physical constitution, but small body size, prolific breeding and easily became tame in captivity could be the reason for them to be demonstrated or live in harmony with human environment . These animals were naturally dominating in biological research. But subsequently guinea pigs from North America were introduced in experimental medical research as they are very sensitive to a number of human pathogens.

Rabbits become an experimental animal because of its easy accessibility being a domestic animal research for fur and flesh. Further it is a prolific breeder and relatively having a huge size compared to the earlier mentioned species or animals. It was first selected for therapeutic trials and how used for presence of pyrogen in fusion fluid. The scientists are utilizing this animal also for raising hyper immune sera for research purposes. Normally birds like fowls are used to test the agents causing poultry disease. Dogs and cats came to experimental purpose because they are domesticated. They cooperate well because of their good human relationship.

Further, they are also having more or less similar physiology as man. They can also be used in experimental surgery. Since primates has close genetical relationship they are also adapted as experimental studies? However, their uses are restricted because they are difficult to be handled. Laboratory animals are used for various purposes which include fundamental biological research, applied medicine, research and diagnosis.

BLOOD COLLECTION TECHNIQUES

Normally for diagnosis blood should be collected from different animals which are ill naturally or an experimental inoculation. Venus blood is commonly drawn out from visible veins. But the site and accessibility of veins differ between animals.The blood volume is normally proportional to the body weight of the animal. For hyperimmune serum harvesting an animal should be bled only $1/10^{th}$ of the blood volume safely without any determental effects to the animal.

ADMINISTRATION OF INOCULUM

Site for various Routes of Inoculation

1. Intravenous
2. Intramuscular
3. Subcutaneous
4. Intradermal
5. Intra peritoneal

COLLECTION OF CLINICAL MATERIALS

(i)Blood for isolation of microbes as serology

Blood can be obtained from intravenous route and inoculate appropriate quantity to the selected media to the suspected organism for which blood was taken.

(ii)Blood smears

Smears may be prepared from peripheral blood collected by cutting the tips of the tail is rodents or from car veins of guinea pigs.

(iii)Tissue impression Smear

It is taken from the cut surface

(iv) Faeces

(v) urine

CELL CULTURE TERMNOLOGY

Anchorage dependence: Requiring attachment to a solid substratum for survival and growth.

Cell Density: The number of cells per unit area of the monolayer.

Cell culture: *in vitro* growth of the cells that in *vivo* no longer get organized in to tissue of their natural origin.

Cell line: A cell line is a populations of cells derive from animal tissue and grow in *vitro* by serial subcultivations for indefinite periods of time with a departure from the chromosome under characterizing its source.

Cell strain: Cell strain is a population of cells derived from animal tissue, subcultivated more than once in *vitro* ,and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin.

Clone: A population of cells derived from a single parent cell by mitosis.

Confluence: The culture situation where all the cells are contact all around their periphery with other cells and no available substratum is left uncovered.

Contact inhibition: Inhibition of cell membrane ruffling and cell motility when cells are in complete contact with other adjacent cells as in confluent culture. Often precedes cessation of cell proliferation but not necessarily related.

Density limitation of growth: Inhibition of mitotic cell division correlated with an increase in cell density to a particular number. This number varies from one cell line to another.

Doubling time: Doubling time generation time is the time it takes for an entire culture to double in number. If all cells are dividing then this time is a measure of overall metabolic efficiency of the population.

Epithelial cell: Cells of epithelial layer origin which in turn is derived from the embryonic endoderm and ectoderm. Often used more loosly to describe cells of a polygonal shape with clear sharp boundaries between cells. Pavement like appearance at confluent state. More correct, this should be termed epitheloid or epithelial like.

Established cell line/continuous cell line: A cell line that has the potential to be subcultured indefinitely in *vivo.*

Explant cells: Cells/tissue/organs removed from its normal environment (in *vivo*) and transferred to an artificial medium (in *vitro*) for growth.

Fibroblast cell: A proliferating precursor cell of the matured differentiated fibrocyte. Often used to describe any cells derived from differentiated mesoderm; usually spindle shaped (bipolar) or stellate (multipolar) and arranged in parallel arrays at confluence if contact inhibited. More correctly this should be termed fibroblastic or fibroblast-like.

Finite cell line: A culture which has been propagated by subculture but is only capable of limited number of cell generation in *vitro* before dying out.

Growth factor: A specific substance that must be present in growth medium to permit a cell to multiply.

Growth curve: The change in the number of cells in growing culture as a function of time.

Hybridoma cell: A single mononucleated (synkaryonic) hybrid cell derived through fusion of an antibody forming lymphocyte and a myeloma cell. When this newly created hybrid cell is subcultured, the clones are referred to as a hybridoma cell line.

Idiogram: The arrangement of(incase of genetic analysis of a cell)the chromosome in order by size and morphology so that the karyotype may be studied.

Karyotype: The distinctive chromosomal complement of a cell strain.

Microcarriers: These are different types of beads coated with charged groups to which anchorage dependent cells get attached and grow in the suspension of beads.

Mitogens: Substance which provoke cell division (mitosis).

Monolayer:A single layer of cells growing on a substratum (Petridish, culture flask etc).

Neoplasia:A new, unnecessary proliferation of cells giving rise to a tumor.

Organ culture: The maintenance of growth of organ primordial, or the whole or parts of an organ in *vitro* specifically designed to allow differentiation and maintenance of the original architecture and function.

Passage: The process of transferring cells from one culture vessel to another. This term is synonymous with subculture.

Passage number: The number of times a culture has been subcultured.

Plating Efficiency: Plating Efficiency is the number of cells that grow up into colonies per 100 cells inoculated, when the inoculum is sparse enough to permit separate colonies.

Plasmids: Cytoplasmic, autonomously replicating chromosomal elements found in bacteria.

Primary culture: The first in *vitro* culture started from any explant taken directly from an organism.

Saturation Density: Maximum number of cells present per cm^2 (monolayer culture)or per ml (suspension culture) under specified culture conditions.

Secondary culture: Cells of the first few (4-6) passages derived from the primary culture.

Split Ratio: The divisor of the dilution ratio of a cell culture at subculture eg, cells from one dish or bottle divided into four (split ratio of 1-4).

Subconfluence: Less then confluent; all of the available substratum is not covered.

Superconfluence: When a monolayer culture in which the cells proliferate suspended in medium, without being attached to the substratum of the vessel.

Tissue culture: A general term that encompasses and is concerned with the study of cells, tissues and organs maintained or grown in *vitro* for more than 24hrs. Three main branches of tissue culture are: (1) Cell culture ;(2) Tissue culture and(3) Organ culture.

Transformation:A process by which a normal eukaryotic cells is altered irreversibly into transformed cell.

Transformed cell:A transformed cell is one that has undergone a stable, heritable change by exposure from certain viruses, chemicals, irradiation, as well as spontaneous mutations. Transformation is often characterized (but not defined) by the emergence of an established cell line from a primary explant, an alteration in typical morphology, loss of contact inhibition, abnormal karyotype, changes in viral susceptibility, changes in antigenic properties, neoplastic properties and the ability to grow in suspension.

APPLICATION OF CHEMICALS

Amido black: This is used to stain proteins. This can be used as an alternative for Coomassie blue.

Ammonium persulfate (APS): This is used as an initiator in the polymerization of polyacrylamide.

Beta-mercapto ethanol: This is used to reduce the disulfide bonds thereby denaturing the secondary structure of the proteins.

Bromophenol blue: This is used as a tracking dye during protein and nucleic acids gel electrophoresis.

Cesium chloride: This is used in the purification of plasmids by equilibrium centrifugation.

Chloroform: This is used as a deprotening agent in combination with isoamyl alcohol in the ratio 24:1. Isoamyl alcohol is an antifoaming agent. Chloroform is also used with phenol during nucleic acid extraction. Chloroform extraction removes proteins as well as phenolic residues present in the nucleic acid preparation.

Coomassie blue: This is used to stain proteins. This can be used to detect only microgram levels of proteins.

Diethylpyrocarbonate (DEPC): This is an Rnase inhibitor used to inactivate Rnases during RNA extraction.

Ethanol: This is used for precipitating nucleic acids. RNA needs 2.5 volumes and DNA needs 2 volumes of ethanol to be added in the presence of 0.17M NaCl.

Ethidium Bromide: This is used to stain the nucleic acids. The substance contains a planer group that intercalates between the stacked bases of DNA. The UV-irradiation is absorbed by the DNA at 260nm and transmitted at 590nm in the red orange region if the visible spectrum. Ethidium bromide can be used to detect both single and double-stranded nucleic acids (both DNA and RNA).However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is poor. As little as one ng can be detected by ethidium bromide staining.

Formamide: This is used as a denaturing agent in RNA gel electrophoresis and in hybridization.

Glycerol: This is used in gel loading buffer to increase the density to the samples. This facilitates the samples to settle at the bottom of the wall.

Heparin: This is an Rnase inhibitor.

8-Hydroxyquinoline: This is an antioxidant used with phenol to prevent oxidation of phenol. This is also an Rnase inhibitor and a weak chelating agent.

Isopropanol: This is used for precipitating DNA and plasmids. This has an advantage that the volume of liquid to be centrifuged is smaller because only equal volume is added in the presence of 0.3 M Sodium acetate.

N,N,N',N' – tetramethyl ethylene diamine (TEMED): This is used as a catalyst during the polymerization of polyacrylamide.

Phenol / Chloroform: The standard way to remove proteins from nucleic acid is to extract once with phenol, once with a 1:1 mixture of phenol and chloroform, and once with chloroform. The deproteinization is efficient when tow organic solvents are used. Though phenol denatures proteins efficiently, it does not completely inhibit Rnase activity, and it is a solvent for long stretch of poly (A) containing RNA. Both these problems can be overcome by using the above mixture.

Phenol: This is used as a deproteinizing agent during nucleic acid extraction. 8-Hydroxyquinoline, an antioxidant, a partial inhibitor of Rnase and a weak chelator of metal ions, is added to the phenol to a final concentration of 0.1%.

Sodium dodecyl sulfatre (SDS): This is used as a denaturing agent in RNA gel electrophoresis and in polyacrylamide gel electrophoresis. It is alos used to rupture the cell membrance. Being an anionic detergent, SDS attaches anionic group (negatively charged) at regular intervals along the polypeptide chains thereby making the separation only on the basis of mass in SDS-PAGE.

Tichloroacetic acid (TCA): This is used to precipitate proteins and nucleic acids. During protein precipitation the proteins are denatured thereby making the solution into suspension.

Vanadylribonucleoside complex: This is used as an Rnase inhibitor during Rnase extraction. The complexes formed between the oxovanadium ion and any of the four ribonucleosides are transition-state analogs that bind to many Rnases and inhibit their activity almost completely.

Xylene cyanol: This is used as tracking dye during nucleic acid gel electrophoresis.

REAGENTS AND BUFFERS

Calcium-Magnesium free phosphate buffered saline (PBS):

1. NaCl	-	8 g
2. KCl	-	0.2 g
3. Na_2HPO_4	-	1.15 g
4. KH_2PO_4	-	0.2 g

Make up to 1000 ml in double distilled water. Autoclave before use.

Hanks Balanced salt solution (HBSS):

1. $CaCl_2$	-	0.185 g
2. KH_2PO_4	-	0.06 g
3. KCl	-	0.4 g
4. $MgSO_4$	-	0.2 g
5. NaCl	-	8.0 g
6. $NaHCO_3$	-	0.35 g
7. Na_2HPO_4	-	0.048 g
8. Glucose	-	1.0 g
9. Phenol red	-	0.016 g

Dissolve in 1000 ml of double distilled water, filter sterilize and store at 4ºC.

Trypsin : (2.5% stock)

Dissolve 0.25 g of trypsin in 10 ml of PBS. Place in water bath at 37ºC to dissolve with occasional shaking. Subsequently pass through Millipore filter to remove undissolved clumps. The 2.5% stock is diluted 10 times for trypsinization.

REFERENCE BOOKS AND MANUALS

1. Readings in Mammalian cell culture. R. Pollack., Cold Spring Harbour Laboratory (1981).
2. Animal Cell Culture. R. Pollack and S. Pfeiffer, Cold Spring Harbour Laboratory (1971).
3. Experiments with Normal and Transformed cells. R.Crowe., H. Ozer and Dr. Rifkin. Cold Spring Harbour Laboratory (1978).
4. Hand Book of cell and organ culture. D. J. Merchant., R.H. Kahn and W. H. Murphy., Burgess Publishing Company (1969).
5. Culture of Animal Cells. R. Ian Freshney and R. Alan., Liss. Inc. (1987).
6. Microcarrier culture: Principles and Methods, Pharmacia Fine chemicals.
7. Animal cell biotechnology. Vol. I and II, R.E. Spier and J. B. Griffiths, Academic Press (1985).
8. Growth of cells in Hormonally defined media, Book A & B. G. H. Sato., A. B. Pardee and D. A. Sirbasku. Cold Spring Harbor Laboratory (1982).
9. Molecular cloning: A laboratory Manual. J. Sambrook, E. F. Fritsch & T. Maniatis Cold Spring Harbour Laboratory (1989).
10. Cell Biology: A Laboratory Manual, G. Shanmugam, Macmillan (1988).
11. Short Protocols in Molecular Biology. F. M. Ausubel *et al.,* Wiley (1999).