EXPERIMENT NO. 4

TISSUE CULTURE

The cell cultures became commonly used after the introduction of antibiotics and fungistatic substances the use of laminar flow cabinets is also very helpful in perverting the contamination of culture as well as the leakage of virus. However the aseptic precautions are still essential. The finger contamination is possible and this necessitates careful handling of cell culture material. There are antibiotic resistant mycoplasmas, which pose problem. The cultured cells are used for virus isolation virus titration, vaccine production and biochemical studies. Since 1949 when Enders, weller and Robbins reported that poliovrus could be grown in non-neural cells with the production of cytopathic changes, large number of unknown viruses were isolated in cell cultures. The advantage of cell culture over chicken embryos or experimental animals are

1. Cell cultures can be produced in large quantities and stored at -70℃.
2. Cell cultures give a clear cytopathic effect.
3. Cell cultures can be grown in chemically defined medium, which is free from antibodies or other infections.
4. Cell cultures can be radiolabile to study the details of virus multiplication.

Glasswares and equipments for tissue culture work

Various types of material are used in cell culture as described below.

Glass Ware

The material must commonly used for growing cells is glass since it is readily available and is cheap and can be used repeatedly. All the glass wares used in cell culture should be of neutral type and the borosilicate glass is highly suitable for cultivating cells. The new glass wares may give a poor growth of cells even after careful washing but start giving satisfactory results when used and once or twice. No satisfactory explanation is available but it is assumed that residual toxic ions inhibit the growth of cells at first time but are sufficiently removed after ward to permit a good growth.

The following are the glass wares with their specification which are generally used in cell culture work.

Milk dilution Bottles (160 ml capacity)

This is used for growing mololayer cell culture is which stock virus is prepared. It is also suitable for plaque assay of viruses. The cell culture media may be also kept is them so that at one time one bottle is taken and the whole medium is consumed. Ten-ml cell suspension is put in it.

Roux culture Bottle (1000-ml capacity)

It is suitable when virus production on large scale is desired. One hundred ml cell suspension is put is these bottles.

Culture tube without rim (15 x 150 mm)

They are use for growing the cell culture either on their surfaces or on the cover slip inserted in them one ml or two ml cell suspension is added in them.

Leighton tubes

These are tubes as item no 3 except that a flat surface is made at inner side of the closed end of the tube so that coverslip is put in that flat area. It is quite convenient since the full cover slip may be allommodated.

Culture tubes, Round bottom with screw cap and Rubber liner (15 x 155 mm)

It is used to grow the monolayer culture and requiems one ml cell suspension.

Petridish culture (50 mm diameter)

It is suitable for growing monolayer culture and is of much use when doing plaque assay of the virus.

Trypsinizing flask

It is a special flask with corrugated wall and a spout and is suitable for separating the cells from tissue under the influence of enzyme trypsin.

In addition, several other types of glasswares like conical flask, beakers, pipettes etc are required. They are available in different capacities and the catalogue of corning should consulted for their specifications.

Plastic vessels

Polystyrene vessels and other items are now gaining wide use in cell culture and have been found highly suitable. They are suitably treated for cell

culture growth and are presterilized so that they can be directly brought in use in the laboratory, this saves much of the time and investment in washing and preparation of glass ware in the laboratory.

Stoppers for culture vessels

Rubber stoppers are used for various culture vessels such as tubes flasks, bottles, etc. They should be made from high quality rubber (i.e. silicon rubber) since the toxic substances released by inferior quality rubber are harmful to the cells as luckily to be spoiled in subsequent autoclaving they are available in different numbers like 1, 2, 3 etc. which fit into the mouth of glassware's of corresponding dimensions.

Rubber tubing

The same considerations as described for rubber stoppers apply to rubber tubing also silicon rubber tubing is highly satisfactory.

Equipments

The equipments used should be of a good quality. The following equipments are required in virus laboratory.

❖	Deepfreez
❖	Refrigerator
❖	Ultracentrifuge
❖	Refrigerated centrifuge.
❖	Lyophilizer
❖	Hot air oven
❖	Autoclave
❖	Water both
❖	Incubator
❖	Magnetic stirrer with Teflon coated magnetic bar
❖	Filters
❖	Vacuums pump
❖	Florescent microscope
❖	Inverted microscope
❖	Instrument sterilizer
❖	Laminar flow cabinets
❖	Ultra violet lamp

Appliances

Scissors, curved and straight, scalpels, forceps, curved and straight, aprons, face masks, hand gloves.

WASHING PROCEDURES Washing of new glass wares

1) New glass ware contains toxic products derived from manufacturing producer and packing they should be washed carefully.
2) Autoclave at 15 lb pressure for 50 min to 1 hrs.
3) Wash several times with tap water.
4) Immerse in a 4% solution of hydrochloric acid in water over night.
5) Rinse in tap water.
6) Immerse in detergent solution (soap powder or teepol) over night.
7) Scrub mechanically.
8) Rinse in tap water several times. To remove the detergent solution.
9) Rinse thrice in metal distilled water.
10) Rinse thrice in triple glass distilled water.

Washing of used glass wares

1) Scrub the glass wares with 10 percent soap solution.
2) Wash thoroughly with tap water.
3) Rinse in 0.05 percent HCI. Solution to neutralize the alkalinity of the detergent.
4) Wash thoroughly with tap water.
5) Rinse thrice in metal distilled water.
6) Rinse thrice with triple glass distilled water.

Washing of Rubber corks (Bungs)

The above mentioned procedure is followed for those which are in use. The new rubber corks received from the firm are subjected to the following treatment after autoclaving at 15 lbs pressure for 30 minutes.

1) Boil for 15 minutes in 20 percent sodium bicarbonate or sodium hydroxide solution.
2) Rinse in hot tap water
3) Boil for 15 minutes in 20 percent hydrochloride acid solution.
4) Rinse in hot tap water.
5) Rinse thrice in metal distilled water.
6) Rinse thrice in triple glass distilled water.

Washing of filters

It is ideal that seitz filter pads should be washed free of alkali and asbestos fibers by passing considerable amount of sterile glass distilled water.

Sintered glass filters should be cleaned with strong acids after use. Concentrated sulfuric acid to which few crystals of sodium nitrate and sodium chlorate have been added is passed through the filter. After this filter should be rinsed with a large volume of glass distilled water.

Pipettes

1. Wash in tap water
2. Boil in appropriate detergent solution for 10 min.
3. Wash in continuous flow pipette washed for 4 h.
4. Rinse in de-ionized water

Syringes

1. Pour off disinfectant and rinse in tap water.
2. Boil in appropriate detergent for 10 min.
3. Rinse in tap water
4. Boil in deionized water.

Siliconed glass wares

1) Remove the silicone grease from the glassware with benzene.
2) Place the glass ware in grease remover for a few hours.

Grease remover

50% potassium hydroxide	50 ml.
Methanol	850 ml

Do further washing in the normal ways as described for used glasswares.

Washing of metallic appliances

All the appliances such as scalpels, scissors, forceps etc may be boiled in 10% soap solution and then washed as described for glass wares.

Drying and preparation of glasswares and apparatus.

All the materials after washing should be immediately dried in a hot air oven at 60-70℃. The tubes, flasks beakers etc shou ld be kept inverted so that water comes out quickly and drying is completed soon.

After drying, the mouths of the vessels are plugged with cotton wool wrapped in musclen cloth and then wrapped with aluminum foil and sent for sterilization.

Sterilization

The purpose of sterilization is to destroy all the living organisms, which could be the source of contamination. Two methods are used for sterilization of glass wares and apparatus.

Dry Heat

Sterilization by dry heat is done in a hot air oven it is a method of choice for all glassware's and apparatus which do not contain any material which could be destroyed the sterilization is done at 160℃ for 60 min or at 180 for 30 mia

High pressure steam

High pressure steam sterilization is done in the autoclave. All the apparatus which cannot be sterilized by dry heat are sterilized in the autoclave the sterilization should be done at 15 lb/in^2 pressure for 20-30 min.

Media and Reagent

The medium is the most important factor in culturing the cells in vitro. The function of the medium is to provide physical conditions such as pH, O2, CO2, osmotic pressure, and nutrition to the cells in the form of chemical substances such as amino acids, carbohydrates and vitamins. The water used in the preparation of the medium for dissolving these ingredients should be very pare, preferably triple glass distilled. An ordinary medium may contain lactalbumin hydrolysate, yeast extract and serum in a balanced salt solution. The serum is one of the most important growth promoting substances and is essential for the satisfactory growth of the cells. The fetal calf serum has been found to be quite satisfactory and essential for cell lines for primary cell

cultures, the serum collected from a young calf may serve the purpose. For cell lines chemically defined media which contain all the required salt, amino acids, vitamins, etc are used along with the fetal calf serum.

Distilled water

For cell culture work double or triple glass distilled water, should be used. It is better to use metal distilled water for putting into the glass distillation apparatus so that calcium and other salts do not logg the distillation apparatus readily. It is advisable to add a few crystals of potassium permanganate to tap water before commencing distillation so that they may oxidize the steam volatile organic compounds which might otherwise be carried over in to the distillate.

Check the purity of the distilled water by the conductivity test it should have conductivity below 1 PPM of sodium chloride.

Demineralised or de ionized water prepared in de ionize units whose inner surface is made of good quality stainless steel may also be used.

Preparation of solutions

Phosphate buffered saline (PBS)

Solution A

Sodium chloride	8.0 gm
potassium chloride	0.2 gm
disodium phosphate	1.15 gm
Monopotassium phosphate	0.2 gm
glass distilled water	800 ml

Solution - B

calcium chloride	0.1 gm
glass distilled water	100 ml

Solution C

magnesium chloride 6 H_20	0.1 gm
glass distilled water	100 ml

Mix solutions A, B and C together and sterilize in autoclave

2. Hanks balanced salt solution (HBSS)

Sodium chloride	8.0
Potassium chloride	0.4
Calcium chloride	0.14
Magnesium sulphate	$7H_20$ 0.1
Magnesium chloride	$6H_20$ 0.1
Disodium phosphate	$2H_20$ 0.06
Monopotassium phosphate	0.06
Dextrose	1.0
Phenol red	0.02
Sodium bicarbonate	0.35
Triple glass distilled water	1000 ml

Added in form of 0.4 percent solution

To be added before use on form of 1.4 percent solution.

For 1000 ml of Hanks BSS, 5ml phenol red solution (0.4%) and 25 ml of sodium bicarbonate solution (1.4%) is required. Therefore, dissolve the ingredients in a total volume of 970 ml distilled water. For preparing this, take 500 ml distilled water in a flask and put the ingredients after weighing them in the order mentioned. Dissolve completely each of them before putting the next ingredient. After dissolving all the ingredients, bring the volume to 970 ml by adding 410 ml distilled water then add 5 ml phenol red solution. Now the total volumes becomes 975 ml, this may now be distributed in 97.5 ml aliquots and sterilized. At the time of use, appropriate amount of sodium bicarbonate solution is added (2.5 ml of sodium bicarbonate solution to 97.5 ml of HBSS). Antibiotics are added at the time of use.

Hanks balanced salt solution without phenol red

This solution is the same as the previous one except that phenol red is omitted. It is required when medium is prepared for agar overday technique for viruses. This facilitates the staining of the overday with neutral red or any such dye.

Calcium and magnesium free hanks solution

The ingredients are the same as listed for hanks solutions except that contains ao_calcium and magnesium ions. This solution is used for preparing

the trypsin solution in order to enhance cell dissociation during the trypsinization process.

Calcium and magnesium free saline

Glucose	1.0
Sodium chloride	8.0
Potassium chloride triple glass	0.4
distilled water	1000 ml

Dissolve the ingredients in the order mentioned. This solution is required for preparing trypsin solution where calcium and magnesium ions are required.

Trypsin (0.25%)

One gram trypsin (TC;Difco 1.250) is dissolved is 400 ml of calcium and magnesium free solution and is stirred on magnetic stirrer either at 4℃ for overnight or at room temperature for 30 minutes. After this the solution is Seitz filtered and kept at 20℃ in aliquots.

Phenol Red (0.4 or 1%)

One gram or 0.4 gram phenol red is taken in a pestle and mortar and triturated with small amount of N/5 NaOH. solution till it completely dissolves after which the volume is made to 100ml with triple glass distilled water. It is filtered through what man No. 42 filter paper and sterilized at 15 lbs pressure for 15 minutes.

Calf Serum

Blood from heating cow caly is collected and kept in the refrigerator at 4℃. Over night the lot is formed and serum is sepa rated which is them collected then it is kept at room temperature (25℃) for about 1 to 2 hours after which the serum is collected. Now it is centrifuged at 3000 rpm for 30 minutes to remove the red blood cells, it facilities are available, the centrifugation should be carried out in the refrigerated centrifuge to maintain the good quality of the serum and prevent hemolysis of erythrocytes. The serum is then inactivated at 56℃ for 30 minutes se itx filtered and stored at 20℃ in aliquots after testing for its sterility.

Horse serum

Methods of collection and preparation of the serum are the same as described for the calf serum. In many cell cultures horse serum is used when it is suspected that the caly serum might contain some antibodies against the virus, which is being cultivated.

Preparation Of Media Growth Medium

The commonly used growth medium for primary cell cultures such as bovine kidney, lamb kidney, etc. is lactalbumin hydrolysate yeast extract (LAH-YE) growth medium. It consists of the following:

Lactalbumin hydrolysate (Difco TC)	0.5 gm
Yeast extract (Difco Bacto)	0.1 gm
Hanks balanced salt solution	80 or 90 ml

The solution is sterilized by seitz filterization or autoclaving at 10 lbs for 10 minutes. Then the following ingredients are added.

Calf serum	10 or 20 ml
Penicillin	100 unit per ml
Streptomycin	100 ugmperml
Mycostatine	25 unit per ml
Sodium bicarbonate solution (1.4%)	2.5 ml

The total volume of the medium should come to 100 ml therefore the serum and antibiotics are added to make the volume up to 100 ml.

The final pH of the growth medium should be 7.4. For kidney cell culture serum is used at the rate of 20 per cent while embryo fibroblast and testis culture 10 percent is used. For celllines, 20 per cent serum should be used. The pH of the medium should be checked with the help of pH indicator paper (BDH). If the pH is less, add few drops of sterilized sodium bicarbonate solution. If pH is more, then bring it down by adding few drops of dilute hydro chloric acid.

Maintenance Medium

The constituents are the same as listed for the growth medium except that serum is either omitted for added is 2.5 percent concentration. In case of foot and mouth disease virus work, no serum is added.

Siman Virus (S.V) Antiserum

SV5 and SV40 viruses are common contaminants of Rhesus monkey tissue even through the tissue is obtained from apparently healthy animals. When cell culture from monkey is prepared, SV5 and SV40 antisera should be incorporated in the cell culture medium to supress them.

Synthetic Media

Primary cell culture can grow ir a good growth medium containing lactalbumin hydrolysate, yeast extract and calf serum or any other suitable serum in balanced salt solution. Howeve', deploid cell strains and permanent cell lines require more nutritional ingredients e.g. several vitamins and aminoacids and thus media incorporat: ng these ingredients are required. Such media which contain all the nutritional requirement in the form of synthetic ingredients are called as synthetic media. These media are prepared from ingredients whose chemical purity is well known. The commonly used media are listed below.

- Medium 199
- Eagle's minimum essential medium.
- Eagle's basal medium
- RPMI -1640
- Ham's F-12
- BHK 21, Medium

These media are now commercially available in the country in power form. They are dissolved in triple glass distilled water and NaHCCb, antibiotics, and serum are added and sterilized by seitz filtration.

The addition of serum (10-20) is done as per the requirement of the cell line, some cell line such as BhK2, require 1-glutamine and this amino acid is added additionally while preparing the medium. Similarly, tryptose phosphate broth may be added for better growth and maintenance of the cell line, some of the cell lines require fetal calf serum.

HEPES Buffered media

If HEPES (N-2 hydrony ethyl piperazine - N-ethane sulphonic acid) buffered medium is to be sued where CO2 incubators are not available, then HEPES should be used in 20-25 mm concentration along with 10 mm $NaHC0_3$ when earles base is used. The composition of a representative HEPES buffered growth medium for a BHK21 cell is as follows.

Trypton phosphate broth	2000 mg
L-glutamine	300 mg
HEPES	4760 mg
$NaHC0_3$	850 mg
BHK21, medium (powder) with earls base	1000 ml
Distilled water	1000ml
Calf serum	100ml

In the maintenance medium the serum may be reduced to 2-5% or avoided altogether when working with apthovirus.

Preparation of Cell Culture

There are three types of cell culture as defined below

- Primary cells
- Primary cells populations are cells obtained from intact animal tissues and grown on glass or plastic surface for the first time.

Diploid cell strains

Diploid cell strains are primary cell populations that have been sub cultivated several time which retain the normal chromosomes constitution of the tissue of origin, and are capable of a finit number of subcultivation.

Heteropioid cell lines

Heteroploid call cell lines are cell populations that can be sub cultivated indefinitely, which reveal abnormal chromosomes number or morphology, and posses abnormal chromosome number or morphology,. and possess other properties that distinguish them from normal cells.

Primary Cell Cultures

Tissue obtained from neonatal or young animals can be used to obtain large quantities of cells. Although these cells do not have long life invitro, they are highly suitable for virus isolation since the various types of cell present in

the primary culture ensure a wide spectrum of sensitivity to viruses while the established cell lines may have a narrow spectrum of viruses to which they are susceptible since embryonic or young tissue cells are capable of undergoing more multiplication cycle in vitro as compared with cells from adult animals, the age of the animal should be as low as possible. The requirement and susceptibility to viruses for primary cell culture is shown in table.

Table

Primary cell cultures, their media requirement and sensitivity to viruses

Primary human embryonic kidney	Eagle's MEM with 10% bovine serum	Epith	Polio, rhinovirus, measles, mumps, entero virus, herpes
Primary rabbit kidney	Eagle's MEM with 10% bovine serum	Epith,	Wide variety of viruses rubella, herpes B, required for testing of all human vaccines produced in primary monkey kidney culture.
Primary bovine kidney	Eagle's MEM with 10% bovine serum	Epith, fib,	Bovine viruses such as FMD, P13, IBR, bovine virus diarrhea.
Primary rat embryo	Eagle's MEM with 10% bovine serum	Epith, fib.	Viruses affecting rodents
Primary monkey kidney	Eagle's MEM with 10% bovine serum	Epith, fib.	Several human viruses, adenoviruses
Primary hamster embryo	Eagle's MEM with 10% bovine serum	Epith, fib.	Adnovirus 12, herpes simplex, influenza A, reovirus
Primary chick embryo	Eagle's MEM with 10% bovine serum	Epith, fib.	Influenza, vaccinia, measles, NDV, poultry viruses
Primary lamb kidney	Eagle's MEM with 10% bovine serum	Epith	Sheeppox goatpox lumpy sken disease
Primary bovine thyroid	Eagle's MEM with 10% bovine serum	Epith	FMD
Primary bovine tasty	Eagle's MEM with 10% bovine serum	Epith	Viruses affecting bovines
Primary mouse embryo	Eagle's MEM with 10% bovine serum or medium 199	Epith, fib.	Viruses affecting mice
Primary chick kidney	Eagle's MEM with 10% bovine serum	Epith, fib.	Viruses affecting chicken

Epith = Epithelial, Fib = Fibroblastic

Chick Embryo Fibroblast Cell Culture

1. Select a 10 day old fertile egg received from a disease free flock and incubated properly. Select only well developed, active embryos with good blood supply, then candle the eggs and select a proper one.
2. Apply tincture iodine on the entire surface of the egg and keep the egg's air seal end uppermost
3. With the help of sterile forceps, break the egg shell at the air sqc, flame the forceps in between.
4. Remove the shell, the shell membrane and the chorioallantoic membrane.
5. Using the curved forceps take out the the embryo by catching it by the peak and place it in a petridish containing B.S.S. like wise remove several embryos using different sets of instruments.
6. Wash the embryo several times with BSS so that it becomes clean.
7. Using a pair of scissors and a forceps remove the head and limbs. Then remove the internal organ by tearing the abdominal wall with the scissors.
8. Transfer the embryo to a beaker containing BSS and wash it several times with BSS so that the blood and other debris are removed.
9. Mince the embryo with the help of a pair of curved scissors.
10. Wash the minced tissue with BSS several times by pouring fresh BSS, shaking the beaker well, allow the tissue fragments to settle and then discard the supernatant.
11. Give a final washing with trypsin solution warmed before at 37℃.
12. Transfer the tissue pieces to a trypsinizing flask containing a teflon coated magnetic bar and pour sufficient amount of trypsin.
13. Place the trypsinizing flask on a magnetic stirrer and run it at a moderate speed.
14. After 15 minute of trypsinization shut off the stirrer, allow the tissue fragments to settle, and then discard the supernatant which process will remove the toxic factors released from the tissue fragments.
15. Pour fresh trypsin solution and continue trypsinization for 30 min after which shut off the stirrer allow the tissue suspension in a centrifuge tube with a funnel with gauze.

16. Carry out such trypsinization 2-3 times depending upon the requirements of the cells or till almost all the tissue fragments are digested.
17. Centrifuge the cell suspension at 1000 rpm for 10 min and give two washing with BSS and finally with the growth medium.
18. Suspend the cells in 100 ml growth medium.
19. Count the cells and adjust the cell density at 10^5 cells / ml.
20. Distribute the cells in cell culture vessels.
21. Incubate at 37℃ in the incubator monolayer for ms in about 3-5 days. If required, change the medium on the third day.

Chick Embroyo Kidney Cell Culture

1. Select a 16 to 20 days old developing chick embryo derived from a disease free poultry flock.
2. Take out the embryo as described below for chick embryo fibroblast culture and put it in a petri dish containing BSS.
3. Dissect the embryo with the help of a pair of scissors and a forceps by making a medium incision on the abdominal wall. Push the visceral organs to one side so as to expose the kidney and collect it similarly, collect the kidney of the other side.
4. Wash the kidneys several times with BSS.
5. Cut the cortical portion into small fragments.
6. Wash several times with BSS and finally with trypsin solution.
7. Transfer the contents to a trypsinizing flask and pour fresh trypsin.
8. Trypsinize for 10 min and discard the supernatant.
9. Again pour fresh trypsin and trypsinize for 30 min, collect the dispersed cells in a centrifuge tube by passing the cells through a gaurge layer. Make two or three collections.
10. Centrifuge, preferably in a refrigerated centrifuge, the cell suspension collected above at 1000 to 15000 rpm for 10 min. Resuspend the cell pellet in the growth medium without serum and again centrifuge. Thus give two washings and finally resuspend the cells in a measured volume of growth medium containing serum so as to give a final concentration of about 5 x $10\sim^5$ - 10^6 cells per ml this may be achieved by counting the viable cells in a hemocytometer after trypan blue

staining or approximately dilute the cell 1:250 to 1:300 (V/V/) by packed cell volume (PA/) of the cells.

11. Dispense the above cell suspension glass vessels of different size and volume depending upon the requirement of the culture. Use the type of vessel shown in table. Allow roughly 1:10 ratio of tissue cell suspension volume and the internal volume of the vessel to provide sufficient oxygen and ideal environment for the cells to grow.

12. Incubate the vessels at 37℃ by keeping them fl at (bottles, flasks) or in an inclined position (tubes in cell culture racks) or on a roller drum (bottles and tubes), run at a speed of 11-12 rpm observe the cultures after 24 hours under inverted microscope and daily there after for formulation of cell monolayer. Usually a confluent monolayer is formed in 4-6 days. Change the growth medium on the third or fourth day if necessary.

Vellels used for cell culture

Vessels (glass or plastic)	**Volume of cell suspension to be dispensed**
15 x 125 mm culture tube	1 ml
petri dish (30 mm diameter)	2 ml
160 ml milk dilution bottle	10 ml
50 ml flask	5 ml
100 ml flask	10 ml
1 roux flask	100 ml
Microtiter plate	0.05 ml/well

Secondary Culture

When the cell culture monolayer grown in bottles are subcultured in to other bottles they are called secondary cultures. Usually secondary cultures are better when the cytopathic effect (CPE) and the inclusion bodies are to be observed by staining since the cells are spread up better as compared with the primary culture. In addition several tissues such as bovine kidney, lamb kidney, monkey kidney, embryonic kidney etc are not easily available, it is advantageous to subculture, the primary culture to prepare secondary cultures so that additional bottles or tubes and cells suspension for microplates are available and more study can be done with the limited cells available.

1. Select a bottle having a complete monolayer and decant the medium.
2. Wash the monolayer with CMF - BSS Thrice.
3. Add 2 ml trypsin (0.25%) and spread on the monolayer surface by gentle rotation.
4. Decant the trypsin leaving about 0.5 ml in the bottle.
5. Incubate the bottle at 37℃ for 1-5 min. Watch i n between for the effect of trypsin on the monolayer. As the cells would become round due to the action of trypsin, adjust the time of incubation accordingly.
6. Decant the excess trypsin.
7. Add 10 ml growth medium and detach the cells by uniform pipetting examine the bottle under microscope for complete detachment of the cells.
8. Add sufficient volume of the growth medium so as to prepare three bottles. If desired, count the cells and make dilutions accordingly. Similarly, if desired, centrifuge the cells and again resuspend so as to completely remove the traces of trypsin the serum in the growth medium inactivate the trypsin and poses no problem.
9. Incubate at 37℃ and observe for cell multiplica tion and monolayer formation.

Cell Lines

The greatest advantage in using a cell line is that it gives an unlimited amount of cells by continues passages. The cell lines always grow uniformly and can be grown on glass or plastic surfaces as monolayer as well as in suspension. The subculturing of cell lines is done in the similar way as described for. secondary cultures. The established cell lines, their media requirement and virus sensitivity are given in table.

Table

Established cell lines, their media requirement and virus sensitivity

Species	*Cell line*	*Derivation*	*Growth medium*	*Split ratio*	*Morphology*	*Viral susceptibility*
Cattle	MDBK	Bovine kidney	MEM, 10% fetal bovine serum (FBS), 2 mm glutamine	1:10	Fib.	BVD, IBR, VSV
	IMR-31	Buffalo lung	McCoy's 5A, 20% FBS, 2 mm glutamine	1:2	Fib.	HSV, vaccinia pseudorabies BVD, PI 3, IBR, bovine enterovirus, VSV
Hamster	BHK21/ CI13	Polled kidneys from 1 day old syrian hamster	Eagle's MEM glasgow modified, 10% FBS, 2 mm glutamine, 10% tryptose phosphate broth	1:30	Fib.	FMDV, rabies, pseudorables, Adenovirus, VSV, reovirus, polyoma
Human	Hela	Cervical carcinoma	MEM, 10% FBS, 2Mm glutamine	1:40 Epith.	Poliovirus, Adenovirus	
	HEp2	Carcinoma of larynx	Eagle's basal medium 15% FBS 2 mm glutamine	1:20	Epith.	Poliovirus, Adenovirus, VSV

	KB	Oral epidermoid carcinoma	MEM, 5% FBS, 2mM glutamine	1:20	Epith	Poliovirus, Adenovirus
	Flow-2002	Human embryonic lung	Eagle's basal medium 10% FBS, 2 mm glutamine, 1% non essential	1:2	Fib.	Poliovirus, HSV, RS, sendai, Rhinovirus
Monkey	BSC-1	African green monkey kidney	MEM, 10% FBS, 2 mm glutamine	1:2	Epith.	Polio, VSV, SV40
	Vero	African green monkey kidney	Medium 199, 5% FBS, 2mM glutamine	1:15	Fib.	Semliki forest virus, tacaribe, herpes simplex
Mouse	Clone M3	Melanoma cloudman S91	Ham's F10, 2.5% FBS, 15% horse serum,3 mm glutamine	1:2	Epith.	HSV, vaccinia, VSV, pseudorabies
	NCTC clone L929	C3H/An mouse areolar and adipose tissue	Ham/s F10 2.5% FBS, 15% horse serum, 2mM glutamine	1:4	Neuroblast	HSV, VSV, vaccinia
	3T3	Swiss mouse embryos	Eagle's medium, dulbecco's	1:20	Fib.	Polyoma, HSV, vaccinia
	3T3	Swiss mouse embryos	Eagle's medium, dulbecco's	1:50	Fib.	HSV, VSV, pseudorabies

			modified, 10% FBS, 2 mm glutamine			
Pig	PK15	Clone of pig kidney line PK-29	MEM, 5% FBS, 2 mm glutamine	1:20	Epith.	Hog cholera, FMDV, African swine fever, vesicular exanthema
Rabbit	RK13	Rabbit kidney	MEM 10% FBS, 2 mm glutamine	1:15	Epith.	Rubella, herpes B, HSV, vaccinia

Abbreviations:

Fib = Fibroblast - like

Epith = Epithelium

FBS = Fetal bovine serum

BVD = Bovine virus diarrhea

IBR = Infectious bovine rhinotracheitis

VSV = Vesicular stomatitis virus

FMDV = Foot and mouth disease virus

HSV = Herpes simplex virus

RS = Respiratory Syncytial

P13 = Parainfluenza 3

Micro Cultures

With the introduction of the microtiter system in virology, immunology, and serology, its application has increased manifold and now it has become a routine procedure to use this system for the cultivation of cell cultures and for their application to various purposes. In this system, cells are grown in microtiter plates, which can be used for various purpose.

Micro Carrier Culture

Microcarrier culture is a culture system in which the tissue cells are grown as monolayers on the surface of small solid particles and kept suspended in the culture medium by constant stirring. In this way the characteristics of both suspension culture and monolayer culture are brought together while retaining all the advantages of both culture systems the micro carrier culture method provides on enormous growth surface area to volume ratio and by using microcarriers in simple suspension culture yields of several million cells per milliliter can be obtained. In this culture as in suspension

culture, measurement and control of the environmental conditions such as temperature, pH, O2 and CO2 tension are facilitated. In addition, replacement of the medium and washing of the cells are facilitated.

Suspension Culture

When a large yield of cells of cells is required, the suspension culture is the best method. The cells are growth in suspension without allowing them to attach on the surface of the vessel and this is achieved by constant stirring of the vessel contents, the O2, CO2, pH and other environment conditions are provided to suit the requirement of the cells.

For cultivating cells on a moderate scale a suitable vessel with a plastic enclosed magnet is available, and after adding the cell suspension the vessel is placed on a magnetic stirrer so that the cells are maintained continuously in suspension.

For suspension culture on a large scale, larger size fermenter vessels of several hundred litre capacity are employed with electronic controls for maintaining all the required optimum conditions. Suspension culture are mainly employed for the manufacture of vaccine.

Applications of Cell Cultures

Cell culture, since its introduction, has become an indispensable tool for different kinds of study. Most of the experiments which were conducted in animals and developing chick embryo before are now being advantageously conducted in cell culture in various types of experimentation. A brief outline of the applications of cell cultures in different disciplines is presented below.

Virlogy

The various applications of different ell cultures in virology have already been discussed and would not be repeated here. We cannot now think of working in virology without cell cultures. In addition to the applications already described, there are some additional uses of cell culture such as viral genetics, interferon production, etc.

Immunology

Cell culture has proved a valuable tool for conducting a large numbed of immunological tests such as serum neutralization, immunofluorescence, immunoenzyme technique, etc. The production of immunoglobulin of a very high titer is now a routine laboratory procedure with the use of cell culture

techniques. A large number of cell lines of human lymphoid origin are now available in long-term suspension cultures, which produce immunolobulin. The hybridoma technique has now made it possible to produce monoclonal antibodies of high titer and specificity against a variety of antigens for use in diagnostic and research purposes. Further advances are taking place in this field.

Bacteriology

Several bacteria are known to produce toxins, which can be assayed, and also toxin neutralization test carried out in cell culture. The effect of bacteria in the living cell in culture may be studied by monitoring the various changes taking place in enzymes and other substances.

Parasitology

Some of the fastidious parasites such as theileria and others have been grown in cell culture, which has provided a valuable tool in their study. Cell culture has also helped in cultivating a large amount of those parasites for the purpose of vaccine manufacture. The growth cycle of those parasites may also be studied in the cell culture system.

Mycology

Mycotic toxins may be assayed in cell culture and the effect of the toxins in cells in culture may be studied.

Toxicology

A large number of toxicological studies may be carried out in cell culture. Of special importance is the screening of a variety of substances for their toxic effect in cell culture before conducting experiments in animals, similarly, organ cultures may be used to assay the toxic effect of various substances.

Pharmacology

Several chemotherapeutic agents are first screened in cell culture and then such studies are carried out on animals. The antiviral agents are screened on a large scale in using the cell culture system and those showing some promise are repeated on animal and man. This has provided a good tool for selecting the promising and

effective antiviral agents. In addition, the effect of drugs on specialized type of cells in culture such as muscle cell etc. may provide valuable information on the efficacy of the drugs and their mode of action.

Physiology

Several physiological studies are carried out by cultivating the cells in culture, the development of blood cells is studied by cultivating bone marrow cells, similarly, muscle cells and nerve cells are cultivated for their study in culture, the effect of various factors on these cells may be studied in controlled conditions.

Anatomy

The structure of cells in culture by light and electron microscopy provides a valuable method for understanding their anatomical features. Cells at various developmental stages are studied.

Endocrinology

The envelopment of cell culture technique has enabled endocrinologists to study hormones and expression of specific cell functions at the cellular level.

Cultures have been established from rat pituitary tumor, and clonal strains of rat pituitary tumor cells which serete growth hormone and prolactin into the medium have been serially propagated in culture. Growth hormone and prolactin can be measured in the culture medium or in the cell homogenates by the microcomplement fixation test. The production of hormones by cells in culture can be altered by many factors, which have been found to influence the pituitary gland in the intact animal. The human chorionic goandotropin (HCG) is a human hormone for which a bulk hormone-producing source has been established hi vitro. For this purpose trophoblastic tissue from malignant placental tumor, choriocarcinoma, has been used for culturing the cells. Production of parathyroid hormone and calcitonin has been reported by using organ culture and cell culture, cultures derived from the mammary tissue have been used for the evaluation of milk protein synthesis.

Nutritional Studies

Cells in culture provide an excellent system for their nutrition requirements. The effect of alteration in nutritional ingredients on cell growth and the enhancing effect of growth promoting factors on cells are studied.

Genetics

Genetic defects can be easily studied under controlled condition by culturing the cells in virto. Skin fibroblast cells in culture and amniocentesis provides excellent tools for the study of genetic defects. The effect of mutagenic substances can be studied in this system. Peripheral leukocytes may also be cultured for genetic analysis.

Cancer Research

One of the major applications of cell culture is in cancer research. With the demonstration that Rous sarcoma virus, an oncogenic virus, could be cultivated in chick embryo cell culture and polyoma virus, in mouse embryo cell culture, intensive use of cell culture has been made for studying tumor viruses. The cell culture system has been used for the assay of Rous sarcoma virus and other carcinogenic viruses which produce transformed foci, demonstrates of tumor virus antigens in cells in culture by immunofrorescence or immunoenzyme technique. Even tumor cells have been cultured to demonstrate virus replication and enhanced production of tumor antigens so that the same can be detected using appropriate techniques. For example, in Epstein-Barr virus (EBV) infection the infected leukocytes are cultured and further studies carried out.

Molecular Biology

Molecular biology is a recently developed discipline, which encompasses studies at molecular level and interdisciplinary interaction. Cells grown in chemically defined media provide an invaluable tool for carrying out such studies.