*Biyani's Think Tank*

**Concept based notes**

# Animal Biotechnology

## [B. Sc. Biotech-III Year]

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# Preface

I am glad to present this book, especially designed to serve the needsof the students. The book has been written keeping in mind the general weakness in understanding the fundamental concepts of the topics. The book is self-explanatory and adopts the "Teach Yourself" style. It is based on question-answer pattern. The language of book is quite easy and understandable based on scientific approach.

Any further improvement in the contents of the book by making corrections, omission and inclusion is keen to be achieved based on suggestions from the readers for which the author shall be obliged.

I acknowledge special thanks to Mr. Rajeev Biyani, *Chairman* & Dr. Sanjay Biyani, *Director* (*Acad.*) Biyani Group of Colleges, who are the backbones and main concept provider and also have been constant source of motivation throughout this Endeavour. They played an active role in coordinating the various stages of this Endeavour and spearheaded the publishing work.

I look forward to receiving valuable suggestions from professors of various educational institutions, other faculty members and students for improvement of the quality of the book. The reader may feel free to send in their comments and suggestions to the under mentioned address.

**Note:** **A feedback form is enclosed along with think tank. Kindly fill the feedback form and submit it at the time of submitting to books of library, else NOC from Library will not be given.**

**Author**

# Syllabus
## Animal Biotechnology

**Note :** Question No.1 shall consist of questions requiring short answers and shall cover entire paper. The paper is divided into four sections. Students are required to attempt five questions in all, selecting not more than one question from each section. All questions carry equal marks.

**Section I**

- Introduction to animal tissue culture

  Historical background

  The application of tissue culture

  Terminology

  Stages in cell culture

- Outline of the key techniques of animal cell culture

  Setting up the laboratory

  Culturing Cell

  Maintaining the culture

  Quantification of cells in cell culture

  Cloning and selecting cell lines

  Physical methods of cell separation

  Hazards and safety in the cell culture laboratory

- Animal cell culture media

  General cell culture media design

  Natural media

  Synthetic media

  Further consideration in media formulation

Nutritional components of media

The role of serum in cell culture

Choosing a medium for different cell types

- Characterization of cell lines

Species vertification

Intra-species contamination

Characterization of cell type and stage of differentiation

Microbial contamination

**Section II**

- Preservation of animal cell lines

Variation and instability in cell lines

Preservation of these cell lines

Freezing of cells

Quantification of cell viability

Cell banks

- Hybridism's

The limitation of traditional antibody preparation

The basis of hybrdoma technology

The details of hybridoma technology

Long-term storage of hybridoma cell lines

Contamination

Hybridomas from different species

Human hybridomas

Commercial scale production of monoclonal antibodies

- Large scale animal cell culture

Culture parameters

Scale up of anchorage-dependent cells

Suspension culture

# Content

# Chapter 1

# Introduction to Animal Tissue Culture

**Q.1. Define following :**

**a. Organ culture**

**b. Cell culture**

**Ans.** **(a) Organ Culture :** Organ culture is a three dimensional culture of tissue retaining some or all of the histological features of the tissue in vivo. The whole organ or a part of the organ is maintained in a way that allows differentiation and preservation of architecture usually by culturing the tissue at the liquid-gas interface on a grid gel.

**(b) Cell Culture :** Cell culture is the process by which prokaryotic or Eukeryotic cells are grown under controlled conditions. This specific culture derived from dispersed cells taken from the original tissue of interest. These culture have lost their hysototypic architecture and often some of the biochemical properties associated with it. They can be propagated and hence expanded and divided to given rise to replicate cultures.

**Q.2. Write down the applications of tissue culture brief.**

**Outline :**

**- Monoclonal antibodies**

- **Recombinant proteins**
- **Reconstitution and replacement of damaged tissues and cells.**
- **Gene targeting**
- **Ammic center's infertility and embryo transplants**
- **Cytotoxicity testing**

**Ans.** **Monoclonal antibodies :** These are monospecific antibodies and identical because they of immune cell. In 1950s a range of other products synthesised from animal cells have found commercial application. In 1975 Kohler and Mistein produced the first hybridomas capable of secreting a single specific antibody so that now we are able to produce pure preparation of antibodies with known specificties. Monoclonal antibodies for a given substance have been produced and can be use of detect the presence of that. These are useful in western blot test, immunoflono science test, immunohisto-chemistry and also in so medesewes like caner, acute rejection of didneytran rhemetoid arthrihs, RSV infection in children etc.

**Recombinant Proteins :** Large scale cultures of animal cells are becoming increasingly important in the production of a range of valuable products. In 1986, human interferon Y (which is thought to have anti-tumor activity) produced from lymphoblastoid cells in culture was licensed as a therapeutic agent.

**Reconstitution and replacement of damaged tissues and Cells :** Tissue grafting may be necessary. When tissue has been lost or has become dysfunctional for whatever reason.

Living equivalents that survive upon transplantation in animals have been constructed from isolated cell of human skin, thyroid and blood vessels.

Vescular tissue is particularly important since any reconstituted tissue or organ will only survive and develop if it is in contact with blood supply. A blood vessel model reconstituted from collagen and cultured vascular cells has been studied in depth.

Parkinson's disease is a nurological disorder which course tremor, rigidity and disturbanus of posture. Chemical analysis of brain tissue

involved in some types of Parkinsonism has revealed a marked depletion of a neurotransmitter called dopamine and this finding hers led to attempts to introduce cells making the missing substances.

Recent developments include the replacement of cells in the brain with neural cells from another source by neural transplantation. There are 2 source of tissue which have been used firstly human embryonic neural tissue on secondly extracts of the patients own adrenal medulla. This second method known as autografting.

**Gene Targeting :** Various advances have been made in introducing foreing genes by insertional mutagensis into cultured skin and blood vessel cells.

Amnountesis infertility and embryo transplantation. Genetic abnormalities of foetures may be identified by culturing cells colled from the amnion during early poyugnancy. Some of the amniotic fluid is removed by a process called amnifocentesis and the cells are cultured to provide enough material for chromosome analysis.

**Cytotoxicity testing :** Tissue culture has been used to screen many anti-cancer drugs since the demonstration in 1950 of clear correlation between invitro and invivo activities of potential chemotherapeutic agents. Explants of human tumour tissue grown in vitro can be tested for chemosensitivity in order to tailor the patients chemotherapy to suit the individual patient and tumor.

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# Chapter 2

# Outline of the Key Techniques of Animal Cell Culture

**Q.1.** **Write down the method of separation of cells by cytoflowmetry.**

**Ans.** The use of flowcytometry for rapidly counting and analyzing cells was developed by H.J. Fulwyler and L.A. Herzenberg. Typically the cells to be analyzed are first stained with a fluorescent dye (eg. ethidium bromide, which combines with the nuclear DNA) or treated with fluorescent antibody that binds to protein in the cell surface. The cell suspension is then pumped through a small orifice and into the center of a narrow horizontal channel in the "flow cell" assembly. Pumped into the same channel through a second small opening at one end is "Sheath fluid" which serves to sweep the cell suspension away from the measuring orifice and out of the flow cell assembly through a third opening. A beam of light from a laver is directed at the measuring orifice where it excites the fluorescent material in each emerging cell causing the cell to emit a flash of fluorescent light. The fluorescence is detected by a system of photomultiplier tubes that relay electrical signals to the instrument, analyzing system. The number of fluorescent light pulse detected by the photomultiplier tubes is directly related to the number of cells passing the measuring orifice. Moreover the magnitude of each pulse varies in proportion to the quantity of fluorescent material associated with each cell and this provides still another measurement parameter. For eg. if the fluorescent pulses are produced by the cell's

DNA, then not only are the cells counted but a measurement is also made of each cell's DNA content. Using flow cytometrg, thousands of cells can be enumerated and also analyzed in just a few minute.
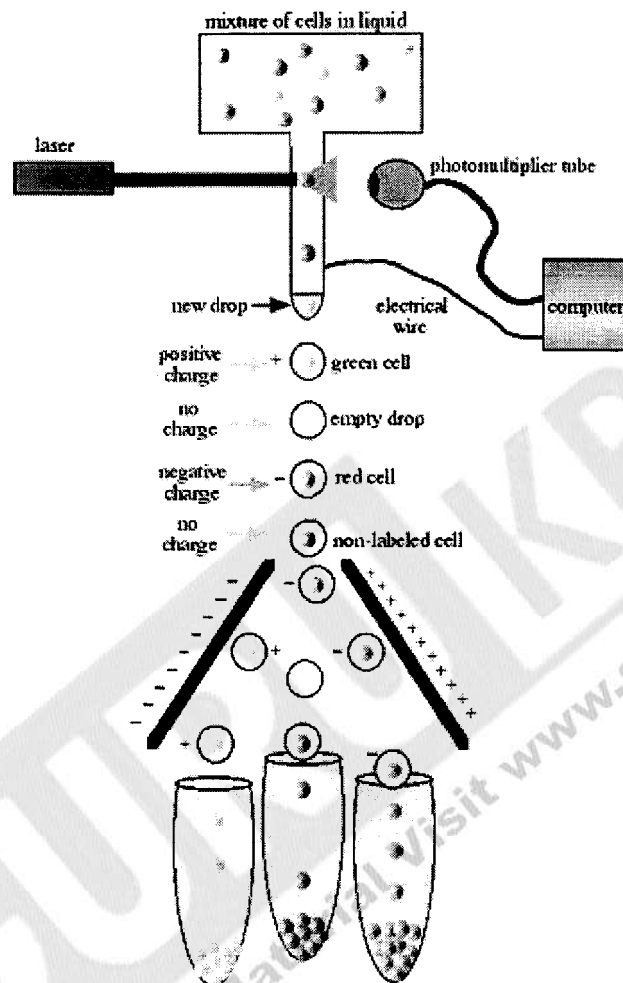


**Q.2.** **Write short notes on the followings :**

**(a) Asecptic techniques**

**(b) Basic equipment used in cell culture**.

**Ans.** **Aseptic techniques :**

**(a)** Some of the techniques may be used to transfer cultures of animal cells.

The basic rules for aseptic techniques for handling animal cell culture and which should be used even if an airflow cabined is available include:

(a) A Bunsen flame to heat the air surrounding by the use of Bunsen burner. This causes movement of air and contaminants upwards and reduces the chances of contamination entering open vessels.

(b) All bottles tops sould be swaped and necks should be clearned with 70% alcohol to clean them before opening.

(c) Flame all bottle necks and pipettes should be flanud by passing very quickly through the hottest part of the flame. This is not necessary with sterile, individually wrapped, plastic flasks and pipettes.

(d) Placing cabs and pipettes down on the bench; should be avovided practice holding bottle tops with the little finger while holding the bottles for pouring.

(e) Work should be done either left to right or vice versa, so that all material to be used is on one side and once finished, is placed on the other side of the Bunsen burner.

(f) Manipulate bottles and flasks carefully. The tops of bottles and flasks must not be touched. Touching of open vessels should also be prevented when pouring. If necessary to pour keep a distance of 5 mm between the two vessels.

(g) Clear up spills immediately and always leave the work area clean and tidy. Dispose of glassware in appropriate bins and bus card used plastic ware in marked polythene bags for autoclaving or incineration. All glassware or plastic-ware used for infections work must always be autoclaved before incincration. Re-usable glassware should be immersed in disinfectant while awaiting transfer to an autoclave.

These rules are by no means exhaustive. The ways in which they are implemented are, however, slightly different in different labs. Learning to apply these rules depend upon gaining 'hand on' experience within a lab.

**(b) Basic Equipments :**

- Cell culture laboratory will need to be furnished with an incubator or hot room to maintain the cells at 60-40$^{O}$C. The incubation

temperature vary according to the type of cells being cultivated. For example insect cells will grow best at around 30$^O$C while mammalian cells require a temperature of 37$^O$C. It may be necessary to use an incubator which has been designed to allow $CO_2$ to be supplied from a main supply or gas cylinder so that an atmosphere of between 2-5% $CO_2$ is maintained in the incubator.

- A refrigerator or cold room is required to store medium and buffers in the tissue culture laboratory. A freezer will be needed for keeping pre-aliquoted stocks of serum, nutrients and antibiotics. Reagents may also be stored at a temperature of -20$^O$C but if cells are to be preserved it may be necessary to provide liquid nitrogen or a -70$^O$C freezer.

- A microscope with normal kohler illumination will be needed for cell counting. An inverted microscope will be needed for examining flasks and multiwell dishes from underneath. Both microscopes should be equipped with a x 10 and a x 20 objective and it may be useful to provide a x 40 and a x 100 objective for the normal microscope.

Additional features such as a camera, adapter and attachments and UV facility may also be required for some purposes. A water bath and a centrifuge with sealed buckets is also necessary. Additional requirements include counting chambers (improved neubauer) or a coulter counter for counting cells, glass or plastic cell culture flasks, graduates pipettes of various sizes, centrifuge tubes and universal containers, disposal Pasteur pipettes, rubber bulbs or automated pipetters for use with pipeetes and precise, calibrated pipetters, e.g. Gilsons, for measuring small volumes from 1-1000 microlitres. More specialised equipment may be needed for particular experiments.

**Q.3.** **Describe the method of preparation of primary culture.**

**Ans.** The first step in primery culture of animal cell is disaggregation of tissue. It is not necessary to disaggregate tissue before culturing. Some embryonic tissue can be cultured simply by leaving the whole tissue on the flask surface and individual cells will simply grow out from the whole tissue

and proliferate. After a few days the original tissue can be removed and the culture medium replenished to allow the new cells to continuing growing. This method works for some tissues but it is not suitable for growing specific cells from a piece of tissue. So we can divide this primary culturel into two catagaries. First one in which tissue do not require enzymatic disaggregation and the second one in which tissue require emymatio disaggregation.

Tissue that do not require enzymatic disaggregation.

Tissue for primary explant can be done in following steps :

- Excess rinsing of tissue by removal of excess blood rinsing with a sterile balanced salt solution (BSS). If the tissue is to be transported it can be kept in BSS or medium;

- Unwanted material such as fat and cartilage is cut off and the rest is chopped finely using a pair of crossed scalpels. It is important to make clean cuts, the use of tearing actions or scissors may damage the cells.

- The cells suspension is transferred to a sterile 50 ml centrifuge tube together with the buffered saline and the cells are allowed to settle out.

- The medium is carefully pipetled off and the pellet is washed in fresh BSS. The cells are allowed to settle again or the suspension is gently centrifugeted at about 1000 rpm for 5 mins.

- The cell pellet is resuspended in 10-15 ml of medium and the suspension is aliquoted into 2 or 3.25 cm$^2$ flasks. If the pieces of tissue are still quite large the suspension may be passed through a sieve before culturing, the cultures are incubated at the appropriate temperature for 18-24 hours. (Note that in describing the cultivation of adherent cells, it is usual to describe the size of the vessels in terms of surface area rather than volume);

- If the pieces of tissue have adhered, the medium should be changed weekly until a significant out growth of cells has occurred.

- The original tissue piece which show outgrowth can then be picked off and transferred to a fresh flask;

- The medium in the original flask is replaced and the cells cultivated until they cover at least 50% of the surface available for growth. They can then be sub-cultured if required.

All the dissection tools, centrifuge tubes and reagents need to be sterilised before use to avoid chances of contamination. With the help of the above steps we draw a flow diagram.

This method is useful for small pieces of tissue such as skin biopsics. Fibroblasts, glial cells, epithelium and myoblasts all migrate out of the tissue very successfully. Some selection occurs because not all cells with adhere to the same extent and some cells rapidly outgrow the others.

**Tissue that require enzymatic disaggregation** : The tissue may be kept whole or may be cut into small pieces as we have discussed. However, densely packed tissues more difficult to digest and prolonged contact with digestive enzymes such as trypsin often causes destruction of viable cells. Large pieces of tissue for enzymatic disaggregation should be cut into small pieces as for the primary explant and digested with trypsin for about 30 min at 37$^O$C. Then addition of serum neutrilise the trypsin. The amount of trypsin require to achieve satisfactory release of cell is tissue dependent and also depends upon the activity of the enzyme preparation. Typically 0.25% w/v trypsin solution is satisfactory.

Trypsin is a proteolytic enzyme which hydrolyses proteins. By adding a lot of protein (in the form of serum), the enzyme begins to hydrolyse these proteins rather than the proteins which bind cells together. Actually we here 'divert' the enzyme to an alternative subtrate so that it is no longer available to attack the extracellular matrix.

Thus once trypsinisation is complete, medium containing serum should be added to neutralise enzyme activity. If trypsin is used to disaggregate whole tissue, the dissociated cells should be harvested after about 30 mins and washed to remove trypsin. The remaining whole tissue may be retrypsinised. An alternative method is to soak the piece of tissue in cold trypsin at 4$^O$C overnight. The cold trypsin penetrates throughout the whole tissue but will have minimal activity at that temperature. The tissue can be disaggregated by increasing the temperature to 37$^O$C for 30-40 mins, then enzyme activity is neutralized by the addition of medium with serum as before.

Collagenase and versene (phosphate buffered saline + EDTA) are also used to disaggregate tissues. Both are gentler in action than trypsin but expensive because larger amounts are required. Collagenase and versene can also be used together with trypsin to allow trypsin to be used at lower concentrations.

We can also use a variety of other hydrolytic enzymes (such as pronase) to disaggregate tissues.

Once disaggregation is complete, the cells can be washed in fresh BSS or medium and seeded out in 24 cm$^2$ flasks. The cell layer can be supplied with fresh medium after 48 hours. This culture is referred to as a primary cell culture.

**Removal of non-viable cells from the primary culture :** If the primary culture is of anchorage-dependent or adherent cells, the non-viable cells can be removed by pouring off the medium and rinsing the cell layer with buffer before adding fresh medium, cell which are not viable will not be able to adhere to the substratum.

If the cells are to be cultured in suspension, the non-viable cells gradually become diluted as the viable cells proliferated. If it is necessary to remove the non-viable cells from the suspension, the cell may be layered onto. Ficoll or 'lymphoprep' and be centrifuged at 2000 rpm for 15-20 mins. The non-viable cells will sink to the bottom and the viable cells can be collected from the medium Ficoll interface.

**Q.4.    Write an essay on Organ Culture.**

**Ans.**    Organ cultures are difficult to prepare in comparison to cell cultures. Once a cell line has been isolated and cloned, preparing fresh cultures simply requires inoculation of fresh medium. Organ culture, however, cannot be propagated. Thus each experiment requires fresh tissue from the donor source. The production of such explants has to be done carefully in order to retain the structural integrity of this tissue.

When cells are cultured as a solid mass of tissue, a number of problems arise which are not an issue with cell culture. In particular, gaseous diffusion and the exchange of nutrients become limiting. To overcome these problems, a number of different techniques have been developed.

Each of these have their limitations.

## Historical development of Organ Culture

One of the earliest tissue culture experiments was performed in 1885, when Roux maintained the medullary plate of a chick embryo in warm saline for a few days. In 1897, Loeb was the first to culture fragments of adult rabbit liver, kidney, thyroids and ovary on small plasma dots inside a test tube. He found they retained their normal histological structure for three days.

This became a standard technique for morphogenetics studies of embryonic organ rudiments and the method was later modified to investigate the action of hormones, vitamins and carcinogens in adult mammalian tissues. The main disadvantage of this technique is that explants often liquify the dot and sink into a pool of liquified medium. For this reason the technique was superseded by the agar dot technique, which uses agar instead of dotted plasma, in the 1940s.

The next major development occurred in 1954, when lens paper (used for cleaning microscope lenses) was used as a floating raft on which cultures were grown. It was possible to study cell-cell interactions by growing different types of tissue on either side of the lens paper.

The use of rafts floating on a fluid medium had a number of disadvantages. The rafts often sank and the tissues were frequently immersed in the medium. This difficulty was overcome by the (Grid-technique) which used metal grids made of wire gauze (and later more rigid expanded metal) instead of lens paper. Tissues are cultured directly on the grid or on strips of lens paper which are deposited on the grid. The method gives tissues a firm support which prevent them from being submerged by the medium. The grids with their explants are placed in a culture chamber filled with medium up to the level of the grid. The method is widely used for embryonic and adult tissues and succeeds in preserving the histological structure of the tissues.

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# Chapter 3

# Animal Cell Culture Media

**Q.1.** **Write a brief account on natural components of media.**

**Ans.** Outline

Amino acids

Nucleic Acid precurorsors

Carbon sources

Vitamins

**Amino Acids:** Essential amino acids which cannot be synthesized by heteroprophic organisms together with cysteine and tyrosine must be incorporated into chemically defined synthetic medium even if a serum supplement is to be used to grow the cells. Requirements may be vary according to the requirements of particular individual. Some times there is a need to incorporate particular amino acids like non-essential to fulfil the requirements. The concentration of amino acids also affect survival and growth of cells. Some times deficiency of amino acids also induces chromosomal damage and increase lysosomal activity and cell death. Imbalance of amino acid concentrations may also produce baryotype changes.

Amino acids consumed most rapidly at the time of lag phase of the growth cycle.

Glutamine plays role as an essential source of energy for carbon. It needs

to be change by regular changing of medium. It also play a good role in the formation of some molecular which are responsible for cell adherion.

**Nucleic acid precursors :** A clenosine, goanosine, cytidine, uridine and thymidine are often added to media particularly if folic acid is in short supply such as in low density cultures.

Medium 199 is usually also deficient in folic acid and needs nucleic acid precursors supplements.

**Carbon Sources :** Glutamine and glucose are very common sources of carbon. If glucose is used, a pyruvate supplement is also necessary.

**Vitamins :** Many vitamins are required to act as cofactors in metabolism.

Retenoids help cells to adhere to substrate and can be used at relatively high concentrations for initial cell adhesion and then at lower levels to maintain the culture.

Deficiency of chotine vitamine causes rounding up of cells and eventual death because it is necessary for incorporation into membrane phospholipids.

Asorbic acid (Vitamin C) is necessary for most cells but should be omitted from media used for growing RNA tumor viruses.

**Polymers :** These are essential for growing cells at low densities or cells which have low planting efficiencies. Polymers improve cell survival by protecting against mechanical damage but do not themselves contribute to growth. Ficoll 400, Dextran 170, Dertran T-500 and methyl/cellulose have all been used as additions to media.

**Q.2.    Write a short note on role of the serum in cell culture.**

**Ans.   Outline :**

- Protein Growth factors
- Hormones
- Attachment and spreading factors
- Binding proteins

- Tatty Acids
- Trace elements
- Inhibitors

**Serum :**

- It contains growth factors, which promote cell proliferation and adhesion factors and antitrypsin activity, which promote cell attachment.

- It is also a good source of minerals, lipids and harmones many of which may be bound to protein (Table: ref Culture of animal cells. R. IANF, energy)

- The serum used most in tissue culture are bovine calf (BCS) and fetal bovine (FBS).

- Human serum is sometimes used in conjuction with some human cell lines.

Serum is used in cell culture medium to provide :

- hormonal factors C. promote cell growth and cellular functions.

- Attachment and spreading factors.

- Transport proteins carrying hormones, mineral, lipid, etc.

**Proteins :**Although protein are a major component of serum, the function of many protein in vitro remain absence.

Albumin found to be important as a carrier of lipids, minerals and globulins.

Fibronection which promotes all attachment although probably not as affectively as cell derived fibrovectin and $\alpha_2$–macroglobulin, which inhibits trypsin.

Fetuin in fetal serum enhances cell attachment.

Transferin binds iron making it less toxic and bioavailable.

Other proteins as yet uncharacterized, may be essential for all attachment and growth.

**Growth Factors :** Most of the growth factors are found in minute amounts

in serum usually in the order of hanograms or picograms per ml. Some like the colony stimulating factors act specifically on cell at a distinct stage of differentiation other act syngergistically on several cell types, for eg.- epidermal growth factor (EGF) promote the proliferation of fibroblasts, epidermal and glial cell. A single cell type may be influenced by variety of growth factors for eg. Fibroblasts responds to fibroblast growth factor. (F), EGF, platelet–derived growth factor (PDGF) and somatomidins. Many of three growth factors are available commercially as recombinant proteins some of which also are available in long form analogs with increased mitogenic activity and stability. PDGF is one of a family of polypeptides with mitogenic activity and is probably the major growth factor in serum. PDGF stimulates growth in fibroblasts and glia, but other platelet-derived factors, such as TGF-β, may inhibit growth or promote differentiation in epithelial cells.

**Hormones :** Insulin is essential for the growth of nearly all cells in culture. It is very sensitive to inactivation by cysteine and has a very short half life so large amount need to be added to the culture medium.

- Glucocorticoid hormones such a hydrocortisone and dexamethesome can either stimulate or inhibit cell growth depending on cell type.

- Other steroid hormones (Such as testosterone and progesterone) and thyroid hormones are also necessary to support some cell lines.

**Attachment & spreading factors :** Most non transformed cells have to attach themselves to a solid substrate in order to grow. Only haematopoetic and transformed cells an multiply without attachment chondronectin in required for the adhesion of chondrocyte and laminin for the adhesion of epithelial cells.

Fibrovectin is also involved in cell attachment.

**Bindin Proteins :** Factors which are of low molecular weight are often carried into cells by binding out transport proteins. For eg. albumin which carries vitamins, lipids and hormones into cell and transfurin which is involved in the binding and transport of iron.

**Fatty Acids :** Cells require varying amounts of essential fatty acids,

phospholipids and cholesterol. Prostaglandins E and $F_2\alpha$ are also involved in cell growth.

**Trace elements :** Copper, zinc, cobalt, manganese, molybdenum and selenium are present and serum and are thought to be involved in activating enzymes and protecting against free radicals which cause damage to DNA.

**Inhibitors :** Serum may contain substances that inhibit cell proliferation. Some of these may be artifacts of preparation but others may be physiological negative growth regulators such as TGF-$\alpha$.

Therefore serum provides an enormous variety of the necessary ingredients to successfully cultivate animal cells.

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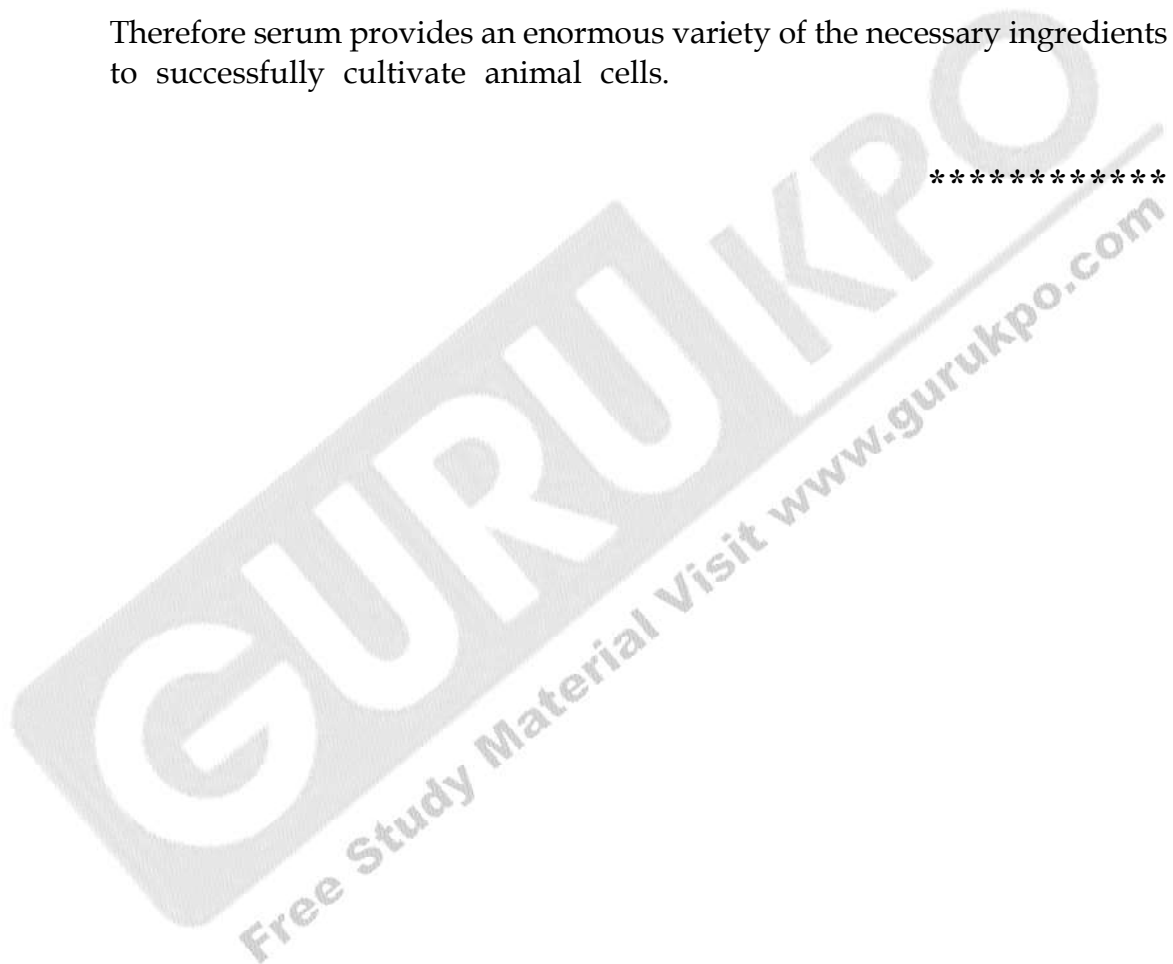
# Chapter 4

# Characterisation of Cell Lines

**Q.1.** **What do you understand by Characterisation of Cell Line. Explain that.**

**Ans.** Once a cell line has been established it has to be characterised. We can describe it in following stages :

**Stage-I : Species verification**

When we work with a specific cell line it in important to know the species of origin of that cell. The species of origin can be confirmed by following methods :

1. Cytogenetics (Karyotype techniques)
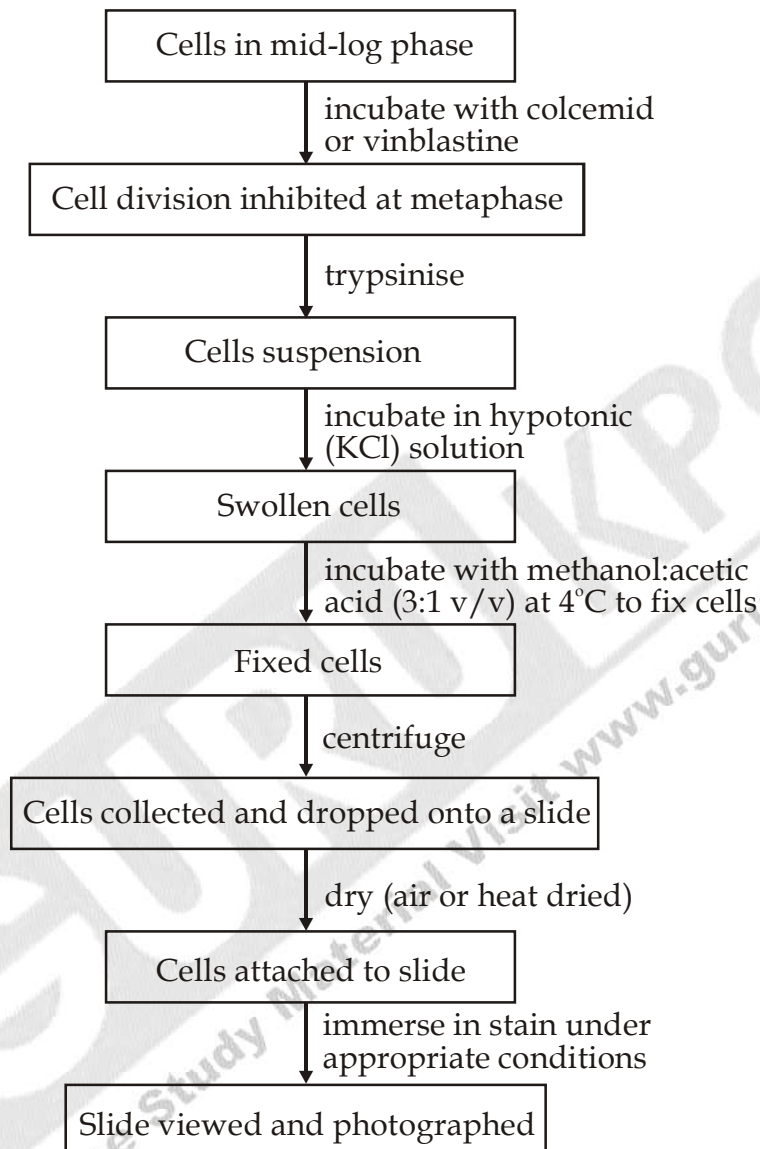
2. Isoenzymology

3. Immunological tests

**Stage-II : Intra species Contamination**

Once the species of origin of a cell line has been confirmed. It may be importance to find out which individual with in that species the cell line is derived from. Some of the genetic techniques are listed below:

(1) Cytogenetics

(2) Recombinant DNA Methods

(3) Hi resolution 2-D electrophoresis

(4)  Allozyme analysis.

(5)  Blood group antigens and HL-A

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┌──────────────────────────────┐
│     Cells in mid-log phase    │
└──────────────────────────────┘
              │  incubate with colcemid
              │  or vinblastine
              ▼
┌──────────────────────────────┐
│ Cell division inhibited at metaphase │
└──────────────────────────────┘
              │  trypsinise
              ▼
┌──────────────────────────────┐
│        Cells suspension       │
└──────────────────────────────┘
              │  incubate in hypotonic
              │  (KCl) solution
              ▼
┌──────────────────────────────┐
│         Swollen cells         │
└──────────────────────────────┘
              │  incubate with methanol:acetic
              │  acid (3:1 v/v) at 4°C to fix cells
              ▼
┌──────────────────────────────┐
│          Fixed cells          │
└──────────────────────────────┘
              │  centrifuge
              ▼
┌──────────────────────────────────────┐
│ Cells collected and dropped onto a slide │
└──────────────────────────────────────┘
              │  dry (air or heat dried)
              ▼
┌──────────────────────────────┐
│     Cells attached to slide   │
└──────────────────────────────┘
              │  immerse in stain under
              │  appropriate conditions
              ▼
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│   Slide viewed and photographed │
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**Stage-III : Characterisation of cell type and stage of differentiation:**

Once the species has been determined, the tissue of origin can be confirmed. The technique used will depend on the cell type, but may

involve functional assays such as milk production, cytogenetic or isozyme analysis or other methods.

**The Organisation of cells in vivo :** Invivo, cells are organised into tissues (groups of similarly specialised cells and associated extracellular matrix) which perform a specific function or group of function. Each kind of tissue is composed of cells with a characteristic size, shape and arrangement. Several types of tissue may unite to form an organ and organ are arranged into organ system. Animal tissues may be classified as epithelial, connective, muscular, blood and lumph.

**Cell division and differentiation** *in vivo* **:** All of the different types of tissue mentioned above develop from a single cell, the fertilized ovum by a process of cell division and differentiation.

Totipotent Ovum

Cell division and differentiation

High serum growth factors low cell density

Pluripotent stem cells can give rise to A, B, C or D

Cell division or differentiation

Unipotent cells, committed precursors each can give rise to only one cell type.

Cell division and differentiation mature differentiated cell.

No cell division, synthesis and secretion of tissue specific products

Hormones extracellular matrix cell-cell interaction.

**Factors needed for differentiation** *in vivo* **:**

1. Hormones

2. Paracrine factors

3. Extracellular matrix

4. Cell-Cell contact

The different cell type which can grow in culture include :

• Fibroblasts

• Epithelical tissue (liver, lung, breast, skin, bladder and kidney) cells.

- Neural cells (Glial cells, neurons although neurons don't proliferate in vitro)
- Skeletal cardiac and smooth mussel cells.
- Skeletal tissue (bone cells and cartilage cells)
- Endocrine cells (Actrinal, pituitary, pancreatlic islet cells)
- Melanocytes
- Many different types of tumor cells.

**Characterisation of cell types** *in vitro* **:** Techniques which are used here depending one the tissues type:

**(A) Morphology :** It is the simplest way to recognise cell types under the microscope. For eg. epithelial cells tend to be regular, polygonal with a clearly defined edge. Fibroblast tend to be bipolar and form characteristic parallel array and whorls visible to naked eyes.

For more specific identification of cell type, there are a number of markers which can be studied. These include:

- Intermediate filament proteins.
- Cell surface antigen.
- Expression of functional properties of the mature cell.
- Analysis of isozymes.

**Microbiological contaminations :** The detection of contaminating micro-organisms detection is an important quality control measure. In cases where cell culture is used to produce a product which is to be marketed.

Bacteria, fungi and yeast are fairly easy to identify and signs of contamination include rapid changes in pH, cloudiness in the medium, extracellular granularity under the microscope and unidentified material floating in the medium. When these symptoms appear culture are generally discarded, especially if frozen stocks are available.

Cell cultures may be examined for these contaminants by sub-culture into suitable media. The media most commonly used for this purpose

are thioglycollate medium, soybean-case in dig it and tryptone soya broth.

It is preferable to grow animal cells in antibiotic free media as the presence of antibiotics may suppress but not eradicate antibiotic resistant contaminants.

Infection of cell lines with viruses can be vary problematic, again, because they often remain undetected. Persistent or latent infection may exist in cell liver and not be noticed until immunological cytological, ultrastructural or bio-chemical tests are applied. In spite of the variety of screening procedures for viruses which exist, latent viruses which do not react in these test will remain undetected, presenting a possible health hazard to the operator and also affecting properties of cultured cells.

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# Chapter 5

# Preservation of Animal Cell Lines

**Q.1.** **Write a short note on variation and instability in cell lines.**

**Ans.** **Outline** :

- Culture conditions
- Selective overgrowth can rapidly alter a cell line.
- A transformed cell can appear in an established cell line.
- Genetic instability can alter the phenotype of a cell line.
- Senescence

**Culture conditions :** Once the optimal conditions for maintaining a cell line have been established these should be adhered to as far as possible. Any changes in the constituents of cell culture medium, batch differences in serum, differences in temperature and gases can alter the growth characteristics of the cell line.

**Selective overgrowth can rapidly alter a alkaline :** In primary cell culture there are a number of different cell types growing at different rates. Some may grow very quickly and overgrow the more slowly dividing or non-dividing cells for eg. if cells are explanted from a colon carcinoma the first culture will contain cells which are mainly epithelial. After subculture, however these epithelial cells, diminish in number and the fibroblast cells take over eventually all the epithelial cells disappear and the fibroblasts from a secondary cell line.

If for a particular experiment, the epithelial cells need to be preserved

then it will be necessary to produce a clone of the cells required and also to freeze down some of the primary cell culture in case disaster strikes and a clone cannot be produced. Once the required clone is obtained, it is vital to freeze down a seed stock which can be used to replenish the working stocks should there get contaminated or lose the particular characteristics required.

**A transformed cell can appear in an established cell line :** Sometimes a cell can become transformed during the life, history of an established or semi-established cell line and take over very rapidly.

The transformation event may enable it to have a much greater growth capacity than the slower growing parent cell line.

**Genetic instability may alter the phenotype of a cell line :** Established cell line have a high incidence of genetic variations partly because the rate of spontaneous mutations in higher in cells which are proliferating rapidly and partly also because there is no mechanism *in vitro* for the elimination of mutants as they appear. These genetic changes and thus the characteristics of the cell line may change altogether. Cells which are to be preserved should be genetically characterised.

**Senescence**

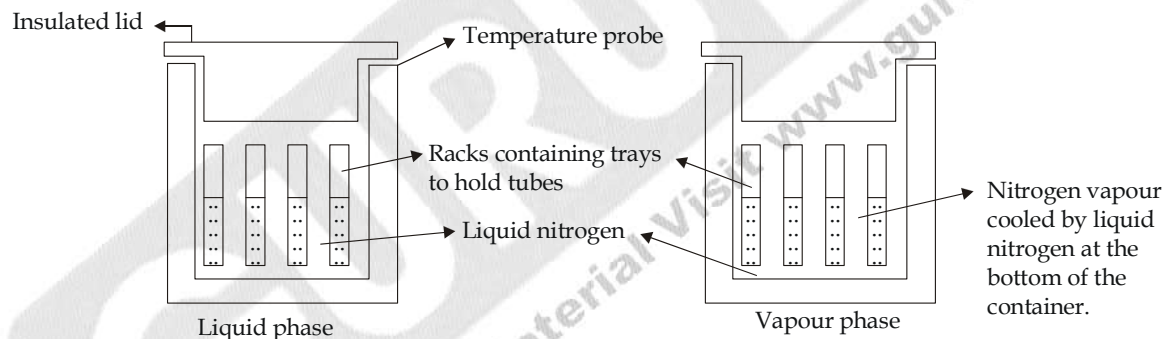Primary explant → Subculture → cell line → Portions
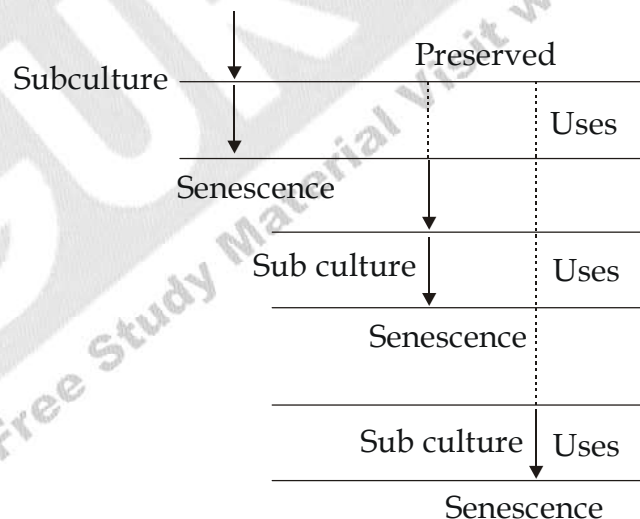
After the problem of senescence cells may lose certain receptors as well as the ability to replicate. By preserving some of the early sub-culture, it is possible to extend the time in which work can be conducted on the cell line.

**Q.2.** **Describe freezing down technique for cell culture.**

**Ans.** Cell can be stored for varying lengths of time at temperature between – 70$^O$C to – 196$^O$C for short term storage for eg. for no more than a few weeks. Cells can be preserved at - 70$^O$C in standard mechanical freezers. For longer time storage, it is necessary to use much lower temperatures and liquid nitrogen is the medium of choice.

Nitrogen containers are also very common in cell banks and laboratories. Cells can be stored in either the vapour phase (-120$^O$C) or the liquid phase (-196$^O$C) of liquid nitrogen.

Storage of -120$^O$C does not significantly reduce cell viability, although for very precious material or for indefinite storage the liquid phase is often preferred.



Insulated lid — Temperature probe

Racks containing trays to hold tubes

Liquid nitrogen

Nitrogen vapour cooled by liquid nitrogen at the bottom of the container.

Liquid phase                    Vapour phase

**Both of the systems have some advantages and some disadvantages:**

Storage in the vapour phase is generally safer. Seepage of liquid nitrogen into the storage tubes (ampoules or cryotubes) does not occur so the risk of explosion is reduced. If liquid nitrogen enters the ampoule, the sudden evaporation of the liquid on removal of the ampoule from the cryostat may cause the ampoule to explode violently.

The risk of the contents of the tube, which may contain infectious material, leaking out of the tube and contaminating the liquid nitrogen and thus,

other tubes is also reduced. For most purposes the vapour phase provides the ideal environment for storing cells.

Liquid phase temperature (-196°C) are advisable when the material is to be stored for very long periods. Particularly precious material should also be stored in the liquid phase so that if a leak develops in the freezer and liquid nitrogen evaporates there is still a time delay before the material is lost.

The cells are stored, ideally at a concentration of around $10^6$- $10^8$ cells/ml in tubes designed specifically for very low temperatures.

These crytobues are preferred by most workers.

**Preparing Cells for freezing :** Cells for freezing are prepared by trypsinisation to detach adherent cells from flask surface.

Cells in suspension are centrifuged at around 100 x g for 5-10 mintues and the pellet is resuspended in small volume (1-2 ml) of storage medium.

- Here specialised medium containing a cryoprotectant (usually glycerol or DMSO) and a high protein concentration (usually extra serum). The formulation of this storage medium has evolved through may trial and error experiments.

**Freezing is stressful :** When viable cells and tissues are frozen a number of stresses are set up within the intra-cellular environment. At around 0°C ice crystals begin to form and these crystals grow as the temperature drops. Because the crystals are made of water alone, the result is that the constituents of the cell which are normally dissolved in water become more and more concentrated as the water freezes. This process of dehydration creats osmotic pressures within the cell which are thought to be the major cause of cell damage and drath.

Rapid chilling results in thermal shock and lead to cell death or injury. Some mechanical injuries to cell membranes during freezing also occur due to ice crystals forming in the extracellular matrix.

**Slow freezing or controlled freezing is vital :** Sophisticated devices are available to control the freezing of cells at a rate of 1°C per minute. Programmmable freezers are useful in laboratories whose main purpose

is cell storage but are generally too expensive for the smaller laboratory. A crude but effective alternative is to place the tubes to be frozen in an upright position in a polystyrene box which can then be placed at $70^{\circ}$C overnight or until the cells have frozen to below – $50^{\circ}$C. They can than be transferred immediately to liquid nitrogen for rapid cooling to -$120^{\circ}$C or $196^{\circ}$C.

**Q.3.**   **Explain why the monitoring of removal of ampoules is necessary.**

**Ans.**   This must be done to ensure that the last ampoules are not removed and the culture lost means it is essential that when the number of ampoules is reduced to a particular critical level, the stock is replenished by repeating the cultivation-preservation cycle.

The number of ampoules produced and the regularity of re-furbishing these shocks is of course, governed by the anticipated and actual use of the stocks.

It is also important to know that who has used stock cultures. If a subsequent problem arises (for *eg.* if the culture is found to be erroneously labelled or contaminated with a virus) the recipient can be traced and altered.

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# Chapter 6

# Oncogenes and Cell Transformation

**Q.1. Write down the three assay suitable for analysing the presence of transformed blood cells.**

**Ans.** We could have choosen focus formation, growth factor requirement and tumorigencity. The anchorage dependency assay would work with non transformed cells as well since the cells cultured from blood are anchorage independent even before they are transformed. This particular criterion of transformation is meaningless with these cells. Morphology would also be a poor choice as these cells are more or less rounded up even in the transformed state.

**Q.2. A non-virulent retrovirus has been isolated which shows a high degrees of specificity for human retina cells. This virus has been genetically manipulated and a Rb-1 gene has been inserted into its genome. Infection of cells *in vitro* with this genetically manipulated virus shows that the Rb-1 gene is expressed, could this virus be used to prevent retinoblasloma production in children who inherit a defective Rb-1 gene?**

**Ans.** If the retinas of the individuals were exposed to high doses to the virus, then many of the retina cells might become infected by the virus. Being non-virulent, the virus would not proliferate but become incorporated

into the host cells genomes. The expression of the Rb-1 genes in these cells would suppress the development of tumours. But there are also same difficulties with this like practically it is difficult to delivering the viruses to the retina cells and ensuring a good infection rate safety is also another difficulty.

**Q.3.**   **Write a short note on Cell Transformation.**

**Ans.**   Transformation is defined as the acquisition by cells of certain phenotypic properties associated with cancer. A number of assay have been developed in order to establish whether cells are transformed and they have generally proved reliable if used in the correct context.

Transformed adherent cells can be indentified by changes, in the control of their growth and behaviour and some of these changes are described in more details below:

**Phenotypic Properties of transformed cells :** The changes to cells when they become transformed can be treated under five headings:

- Ability to form foci *in vitro* culture.
-  Changes in morphology.
- Changes in growth factor requirements.
- Release from anchorage- dependence for cell division.
- Ability to form tumous *in vivo*.

**Focus Formation :** When normal cells are grown in culture they form a monolayer and when this monolayer reaches confluence the cells stop dividing through a process called contact inhibition. Transformed cells loose this property and produce disorganised clusters of cells which grow on top of each other. These are called 'foci'. (Singular, focus) and these are readily recognisable when seen against a background of normal cells growing or a monolayer. This assay is often referred to as the focus forming assay.

**Morphology:** The Morphology of cells changes upon transformation. Usually transformed all tend to round up, probably due to the disruption and reorganisation of cytoskeletal structures.

**Growth for Requirements :** Transformed cells proliferate under conditions in which normal cell do not. When such cells are cultivated *in vitro* they will grow in media containing low serum concentration where as non-transformed cells will not do so.

**Transformed cells become anchorage dependents :** Most cells (with the exception of haematopoietic cells) grow as an adherent monolayer. Adherent cells are said to be anchorage-dependent which means that they need to attach to a substrate in order to proliferate when cells are transformed they acquire anchorage-independence.

Tumorigenicity can be demonstrated in rude mice.

Tumorigenicity: is the ability to generate tumors. This can only be demonstrated in vivo, and is measured experimentally using a special kind of mouse (rude mouse) which is a developmental mutant which has lost its ability to produce thymus gland and therefore has a defective immune defence system. It can therefore serve as a host for the growth of tumorigenie cell originating from any species, since these cells will not be recognised as foreign by the animal's immune response. The rate of tumour formation and the number of tumours formed when cells are injected under the skin of the immune deficient mouse gives an indication of tumorigenicity.

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# Chapter 7

# Hybridomas

**Q.1. Why spleen cells (spenocytes) are more suitable for hybridoma production in comparison to other organs.**

**Ans.** Spleens contain large amounts of lymphoid tissues and have, therefore, many resident B cells. Thus, if we collect spleens, separate the cells (by for example panning), we can harvest many B cells. There is, therefore, a high probability that such a population will contain the desire able B cell type C that is, B cells which produce the desired antibody). Alternatives to spleens would be lymph nods but these are smaller and will be more difficult to handle. Organs such as heart and kidneys contain much lower populations of lymphoates and offer less likelihood of being useful sources of the desired B cell (or plasma cell) type and ore, therefore, rarely used.

**Q.2. Write down two methods of fusing cells.**

**Ans.** Stirring method for fusing cells: in the stirring method, splenocytes and myceloma cells are washed separately by centrifuging at 400 x 9 in serum-free medium and resuspending in 1-5 ml of serum –free medium. The cells are mixed and centrifuged to get the cells into close proximity to each other. All of the medium is then carefully pipetted off and 1 ml

of a 50% solution of PEG 1500, which has been prewarmed to 37$^O$C, is added very slowly to the cell pellet while gently stirring to resuspend the cells. Then 1 ml of serum –free medium is added drop wise over a period of about one minute, while constantly stirring the cell suspension. A further 9 ml of serum free medium are then added over a period of two minutes, still stirring continuously.

The cells are centrifuged again at 400 x 9 for 5 minutes, the supernatant is removed and the pellet resuspended in 10 ml of prewarmed medium with 20% foetal calf serum, OPI solution and selection medium containing aminopterin and hypoxanthine and thymidine (HAT) or 8- azauganine the suspension is made up to 200 ml with medium aliquoted into each well of 20 x 96 well pates and incubated at 37$^O$C in a $CO_2$ incubator. The cell are examined using a microscope after day 2 and are visible to the maked eye by days 4-6. (You might like to draw yourself a flow diagram of these stepls).

**Spinning method for fusing cells :** Cells can also be fused using the spinning method, which requires a lower concentration of PEG. The cells are centrifuged, washed and mixed, then recentrifuged as for the stirring method above and them the pellet resuspended in 0.2 ml of a 30% PEG solution at 37$^O$C the cells are resuspended by gently tapping the base of the centrifuge tube they should not be pipetted. After centrifuging again at 400 x g for 5 minutes, the cells are resuspended in 5 ml of serum-free medium. Again, the tubes are tapped or flicked to resuspend the cells. A further 5 ml of medium supplemented with 20%. Foetal calf serum OPI and HAT as above are added, the cells are mixed gently and made up to a volume of 200 ml and aliguoted into 96 well plates as before.

There appear to be no particular advantages or disadvantages with either method, and the choice of method depends entirely on convenience.

**Q.3.** **Write down the steps in producing hybridomas.**

**Ans.** Four basic steps are there where are as following:

- Immunising mice

- Fusing cells
- Screening procedures.
- Producing the hybridomas.

**Q.4.** **What kind of screening procedures one can use in producing hybridomen.**

**Ans.** Mostly hybridomas grow at approximately the same rate, the tissue culture supernatant containing antibodies all become ready to screen at once. The screening process is very labour intensive so care should be taken before hybridomas are made.

Three types of screening method are routinely used for checking hybridomas.

(a) antibody capture assays

(b) antigen capture assays

(c) functional assays

**(a) Antibody capture assays :** This is the most convenient assay for hybridoma screening. Antigen is bound to solid substratum such as the base of a 96 well microtitre plate or allowed to coat the surface of plastic beads. The culture fluid to be tested is exposed to antigen and allowed to bind for anything from 1 her to over night. Any antibody in the culture fluid which binds to the antigen remains attached to the solid matrix as antigen –antibdoy complexes. While unbound antibody can be washed off. A second antibody which reacts with the antigen-antibody complex is then added. The second antibody is conjugated with either an enzyme which produces a colour change in the presence of an appropriate substrate or with a fluorochrome.

**(b) Antigen capture assay :** The culture fluid from the hybridoma culture is incubate in a microtitre well where antibodies are allowed to attach to the well walls. The culture fluid is then removed and the wells are washed. A solution or suspension of the antigen is then introduced into

the well. After incubation the solution is removed and either the amount of antigen remaining in the well or the amount left in the solution is measured. In this way culture fluids containing the appropriate antibody can be detected.

**(c) Functional Assays :** Antibodies in the culture fluid are assessed by their effect on a function or activity of cells. Usually this involves blocking a reaction by making an essential binding site unavailable or by depleting an essential component of a reaction mix by precipitation of an antigen antibody complex.

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# Chapter 8

# Large Scale Animal Cell Culture

**Q.1.** **Write down an assay on suspension culture.**

**Ans.** Cell lines derived from haemopoietic tissues (for example, H9, Hut 78, Raji) and hybridoma lines normally grow in suspension either as single cells or in small clumps of cells derived from single cells suspension can be scaled up from a flask to a deep tank culture.

**Stirred tank bioreactors:** Typically laboratory scale cultures are produced in spinner flasks they derive their name from the action of a small barmagnet drivers by an external magnetic stirrer. In prineipple, such vessels can be used for volumes from 20 ml to 20 litres. In practice, however, such vessels are difficult to handle once the volume exceeds about 5 litres. For larger volumes we may, therefore, use a stirred tank bioreactor of the type illustrated.

The main advantage is that the stirred tank bioreactor allow for better control of the mixing of the culture properly designed impellers are more efficient mixes than bar magnets the more efficient control of mixing obtained in stirred tank bioreactors has important consequences for maintaining homogeneous culture conditions. There is a lower probability of setting up pH gradients or areas of low oxygen tension in such vessels.

Stirred tank reactors and looped reactors are available upto a

working volume of 8000 litres although for the gas lift types the commercially available vessels are usually produced up to a volume of 1000 litres.

**Changes which occur on scale up :** The scale up from the small spinner cultures used in laboratories to the bio-reactors used on an industrial scale (for example) 1000 litres involves a number of important changes. Hence we will deal with the following five issues.

• the materials used to construct the vessels;

• the method of temperature control;

• the supply of oxygen

• the requirement to be able to sterilise the vessel and its connections in situ.

**Materials used for large vessels :** Generally, the large culture vessels are made of stainless rather than of glass or plastic.

**Temperature control in large vessels :** The advantage of using an a external water jacked it that it does not occupy any of the working volume of the reactor. The main disadvantage of these devices is that the area available for the heat exchange is limited. This is not a major problem with smaller vessels but it can become a problem with very large vessels. Consider for example a cylindrical shaped reactor if the volume of the reactor is doubled the surface area of the reactor is not doubled (you might like to prove this for yourself. Remember that the volume of a cylinder = $\partial r^2 h$ and if we assume that the area for contact with a water jacket is limited to its vertical walls its surface area = $2 \partial r h$. By substituting in values for r and h, you will see that the surface, volume ratio declines as the value of r is increased.
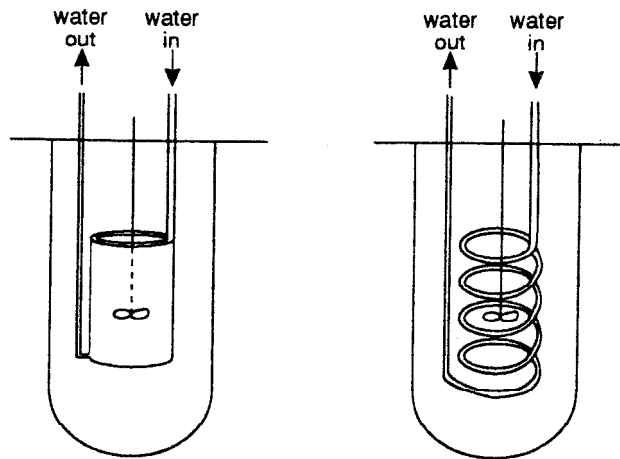
a) cylindrical heat exchange          b) coiled heat exchanger

**Oxygen supply :** There are two fundamentally different ways of increasing oxygen transfer into the liquid. These are by increasing the stirring rates and by sparging. Thus, in sealing up from a small spinner culture to a stirred tank reactor we have to consider whether to increase the stirring rate or to use sparging or to use a combination of both.

**Sterilisation of large vessels :** Large vessels cannot be placed in autoclaves, so sterilisation has to be done in situ. This is usually achieved by connecting the vessel to a steam which injects super-heated steam into the vessel. It is not only essential that the reactor itself is sterilised but also the feeder and outlet pipes must also be sterilised probes (for example for PH, temperature) also require sterilisation. The sterilisation of pipes taps and valves is also achieved using super heated steam. This of course, needs careful reactor design since if any part of the system is not properly sterilised, this may act as a potential source of contamination.

**Looped bioreactors :** A variety of looped bioreactors are shown in the following figure. These reactors have many of the tea tores of stirred tank reactors. But different in that they are designed to induce a cyclical flow of culture around the vessel.

In the gas, lift (air-lift) type, air enters the vessel via a sparger near the

bottom. This creates small bubbles in the medium near the bottom of the draught to be, the medium. Bubble mixture is of lower density that medium alone, so it begins to rise. The movement of medium in the impeller (propeller) type is, of course, driven by action of the impeller whilst in the jet-flow type. The liquid air mixture pumped into the vessel provides the momentum to keep the medium in the vessel well mixed.



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