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(AUTONOMOUS), VELLORE

eRESOURCES

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E CONTENT TITLE : ANIMAL BIOTECHNOLOGY

DEPARTMENT : BIOTECHNOLOGY

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UNIT I

INTRODUCTION TO ANIMAL CELL CULTURE

Animal cell culture basically involves the in vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensible technology in various branches of life sciences.

Historical Background

It was in 1907 Ross Harrison first developed a frog tissue culture technique. He probably chose frog for two reasons being a cold-blooded animal, no incubation is required and tissue regeneration is fast in frog. In 1940's chick embryo tissue became a favorite for culture techniques.

Interest in culturing human tissues started in 1950's after it was demonstrated (by HeLa; Gey) that human tumor cells could give rise to continuous cell lines. Among the various animal cell cultures, mouse cell cultures are the most commonly used in the laboratory.

Terminology in Cell Culture:

The term tissue culture is commonly used to include both organ culture and cell culture.

Organ culture

The culture of native tissue (i.e. un-disaggregated tissue) that retains most of the in vivo histological features is regarded as organ culture.

Cell culture

This refers to the culture of dispersed (or disaggregated) cells obtained from the original tissue, or from a cell line.

Histotypic culture

The culturing of the cells for their re-aggregation to form a tissue—like structure represents histotypic culture.

Organotypic culture

This culture technique involves the recombination of different cell types to form a more defined tissue or an organ.

Primary culture

The culture produced by the freshly isolated cells or tissues taken from an organism is the primary culture. These cells are heterogenous and slow growing, and represent the tissue of their origin with regard to their properties.

Cell line

The sub-culturing of the primary culture gives rise to cell lines. The term continuous cell lines imply the indefinite growth of the cells in the subsequent sub-culturing. On the other hand, finite cell lines represent the death of cells after several subcultures.

Facilities for Animal Cell Culture

While designing the laboratory for animal cell culture technology, utmost care should be taken with regard to the maintenance of aseptic conditions.

Minimal Requirements for Cell Culture

i. Clean and quite sterile area

ii. Preparation facilities

iii. Animal house

iv. Microbiology laboratory

v. Storage facilities (for glassware, chemicals, liquids, small equipment).

Equipment:

Laminar-flow, sterilizer, incubator, refrigerator and freezer (-20°C), balance, $C0_2$ cylinder, centrifuge, inverted microscope, water purifier, hemocytometer, liquid nitrogen freezer, slow- cooling device (for freezing cells), pipette washer, deep washing sink.

Besides the basic and minimal requirements listed above, there are many more facilities that may be beneficial or useful for tissue cultures. These include air-conditioned rooms, containment room for biohazard work, phase-contrast microscope, fluorescence microscope, confocal microscope, osmometer, and high capacity centrifuge and time lapse video equipment.

Culture Vessels:

In the tissue culture technology, the cells attach to the surface of a vessel which serves as the substrate, and grow. Hence there is a lot of importance attached to the nature of the materials used and the quality of the culture vessels. The term anchorage dependent cells is used when the cells require an attachment for their growth. On the other hand, some cells undergo transformation, and become anchorage independent.

Materials used for culture vessels:

Glass:

Although glass was the original substrate used for culturing, its use is almost discontinued now. This is mainly because of the availability of more suitable and alternate substrates.

Disposable plastics:

Synthetic plastic materials with good consistency and optical properties are now in use to provide uniform and reproducible cultures. The most commonly used plastics are polystyrene, polyvinyl chloride (PVC), poly-carbonate, metinex and thermonex (TPX).

Types of culture vessels:

The following are the common types of culture vessels.

i. Multiwall plates

ii. Petridishes

iii. Flasks

iv. Stirrer bottles.

The actual choice of selecting a culture vessel depends on several factors:

1. The way cells grow in culture—monolayer or suspension.

2. The quantity of the cells required.

3. The frequency of sampling for the desired work.

4. The purpose for which the cells are grown.

5. The cost factor.

In general, for monolayer cultures, the cell yield is almost proportional to the surface area of the culture vessel. The flasks are usually employed for this purpose. Any type of culture vessel can be used to grow suspension cultures. It is necessary to slowly and continuously agitate the suspended cells in the vessel.

Treatment of culture vessel surfaces:

For improving the attachment of cells to the surfaces, and for efficient growth, some devices have been developed. It is a common observation that the growth of the culture cells is better on the surfaces for second seeding. This is attributed to matrix coating of the surfaces due to the accumulation of certain compounds like collagen and fibronectin released by the cells of the previous

culture. There are now commercially available matrices (e.g. matrigel, pronectin, and cell-tak).

Feeder layers:

Some of the tissue cultures require the support of metabolic products from living cells e.g. mouse embryo fibroblasts. In this case, the growing fibroblasts release certain products which when fed to new cells enhance their growth.

Alternate substrates as substitutes of culture vessels:

In recent years, certain alternatives for culture vessels have been developed. The important alternative artificial substrates are micro carriers and metallic substrates.

Micro-carriers:

They are in bead form and are made up of collagen, gelatin, polyacrylamide and polystyrene. Micro-carriers are mostly used for the propagation of anchorage-dependent cells in suspension.

Metallic substrates:

Certain types of cells could be successfully grown on some metallic surfaces or even on the stainless steel discs. For instance, fibroblasts were grown on palledium.

Use of Non-Adhesive Substrates in Tissue Culture:

The growth of anchorage independent cells can be carried out by plating cells on non-adhesive substrate like agar, agarose and methyl cellulose. In this situation, as the cell growth occurs, the parent and daughter cells get immobilized and form a colony, although they are non-adhesive.

Contamination, Aseptic Conditions, and Sterilization:

There are several routes of contamination in the tissue culture laboratory (Table 33.1). These include the various materials (glassware, pipettes), equipment (incubators, refrigerators, and laminar-flow hoods), reagents (media, solutions), contaminated cell lines and poor techniques.

TABLE 33.1 Major routes of contamination in a tissue culture laboratory

Equipment and facilities
Laminar-flow hoods
Dry incubators
CO_2 incubators
Humidified incubators
Wooden furniture, benches
Other instruments
Glassware and reagents
Pipettes
Screw caps
Culture glasses
Media bottles
Media and various solutions
Biological materials
Infected tissue samples
Cell lines
Operating techniques
Operator hands, hair, clothing, breathing
Work spaces
Pipetting, dispensing
Operating manipulations

The routes of contamination are mostly associated with the laboratory environment, and operating techniques.

Types of microbial contamination:

Several species of bacteria, yeasts, fungi, molds and mycoplasmas, besides viruses are responsible for contamination. Major problems of conta-

mination are linked to the repeated recurrence of a single species. Despite utmost care taken, no laboratory can claim to be totally free from contamination. It is necessary to continuously monitor for contamination and eliminate the same at the earliest.

Aseptic Conditions:

Maintenance of proper aseptic conditions is necessary to eliminate various contaminants (due to different microorganisms and viruses). The following measures are suggested for minimizing contamination, and maintenance of aseptic conditions.

i. Strict adherence to standard sterile techniques and code of practices.

ii. Checking of reagents and media for sterility before use.

iii. Checking of cultures by eyes, and microscopes (phase contrast) every time they are used.

iv. Use of media and separate bottles for each cell line is advised.

v. Maintenance of clean and tidy conditions at work places.

vi. Personal hygiene of the staff is very important.

Sterilization:

The sterilization procedures are designed to kill the microorganisms, besides destroying the spores.

There are three major devices for sterilization:

1. Dry heat

2. Moist heat (autoclave)

3. Filters.

In the Table 33.2, the sterilization of major equipment, apparatus and liquids is given.

Table 33.2 Sterilization of major equipment, apparatus and liquids used in tissue culture

Sterilization device	*Items sterilized*
I *For equipment and apparatus*	
Dry heat	Glass slides
	Pipettes
	Ampoules (glass)
	Pasteur pipettes
	Instruments
	Test tubes
Autoclave	Ampoules (plastic)
	Apparatus with silicone tubing
	Filters (reusable)
	Glass bottles with screw caps
	Glass syringes
	Magnetic stirrer bases
	Screw caps
	Stoppers (rubber silicone)
II *For liquids and nutrients*	
Autoclave	Salt solutions
	Glucose–20%
	Agar
	Bacto–peptone
	Glycerol
	Lactalbumin hydrolysate
	Phenol red
	Tryptose
	HEPES
	EDTA
	Water
Filter	Serum
	Amino acids
	Vitamins
	Antibiotics
	Bovine serum albumin
	Collagenase
	Glutamine
	Drugs
	NaOH
	Trypsin
	Transferrin

Sterilization by dry heat:

This is carried out at a minimum temperature of 160°C for about one hour.

Sterilization by moist heat:

Certain fluids and perishable items can be sterilized in an autoclave at 121°C for 15-20 minutes. For effective moist heat sterilization, it is necessary that the steam penetrates to all the parts of the sterilizing materials.

Sterilization by filters:

The use of filters for sterilization of liquids often becomes necessary, since the constituents of these liquids may get destroyed at higher temperatures (dry heat or moist heat). Sterile filtration is a novel technique for heat- labile solutions. The size of micropores of the filters is 0.1-0.2 μm. Filters, made from several materials are in use. These materials include nylon, cellulose acetate, cellulose nitrate, polycarbonate, polyethersulfone (PES) and ceramics.

The filters are made in different designs-disc filters, cartridges and hollow fiber. In fact, many commercial companies (e.g. Millipore, Durapore) supply reusable and disposable filters, designed for different purposes of sterilization.

Advantages and Limitations of Tissue Culture:

Advantages of Tissue Culture:

Tissue culture technique has a wide range of applications.

The most important advantages of this technique are listed below:

1. Control of physicochemical environment- pH, temperature, dissolved gases (O_2 and CO_2), osmolarity.
2. Regulation of physiological conditions-nutrient concentration, cell to cell interactions, hormonal control.

3. The cultured cell lines become homogenous (i.e. cells are identical) after one or two subcultures. This is in contrast to the heterogenous cells of tissue samples. The homogenous cells are highly useful for a wide range of purposes.

4. It is easy to characterize cells for cytological and immunological studies.

5. Cultured cells can be stored in liquid nitrogen for several years.

6. Due to direct access and contact to the cells, biological studies can be carried out more conveniently. The main advantage is the low quantities of the reagents required in contrast to in vivo studies where most of the reagents (more than 90% in some cases) are lost by distribution to various tissues, and excretion.

7. Utility of tissue cultures will drastically reduce the use of animals for various experiments.

Limitations of Tissue Culture:

There are several limitations of tissue culture; some of them are given below.

1. Need of expertise and technical skill for the development, and regular use of tissue culture.

2. Cost factor is a major limitation. Establishment of infrastructure, equipment and other facilities are expensive.

3. It is estimated that the cost of production of cells is about 10 times higher than direct use of animal tissues.

4. Control of the environmental factors (pH, temperature, dissolved gases, disposal of biohazards) is not easy.

5. The native in vivo cells exist in a three- dimensional geometry while in in vitro tissue culture, the propagation of cells occurs on a two dimensional substrate. Due to this, the cell to cell interactive characters are lost.

6. The cell lines may represent one or two types of cells from the native tissue while others may go unrepresented.

7. Tissue culture techniques are associated with the differentiation i.e. loss of the characters of the tissue cells from which they were originally isolated.

8. This happens due to adaptation and selection processes while culturing.

9. Continuous cell lines may result in genetic instability of the cells. This may ultimately lead to heterogeneity of cells.

10. The components of homeostatic in vivo regulation (nervous system, endocrine system, metabolic integration) are lacking in vitro cultures. Addition of hormones and growth factors has been started recently.

Risks in a Tissue Culture Laboratory and Safety:

There are several risks associated with tissue culture technology. Most of the accidents that occur in culture laboratories are due to negligence and casual approach while dealing with biological and radiological samples, besides improper maintenance of the laboratory. A broad categorization of risks and the contributory factors is given in Table 33.5.

TABLE 33.5 Risks in a tissue culture laboratory

Category	*Contributing factor(s)*
Maintenance risks	Age and condition of various equipment, leakage of disposals.
Personnel risks	Inadequate training, lack of concentration and interest.
Physical risks	Electric shocks, fire, intense cold.
Chemical risks	Toxicity due to poisons, carcinogens, mutagens, irritants, allergens.
Biohazards	Pathogenic organisms, viruses, genetic manipulations, culture cells and DNA (quality and quantity).
Radioisotope risks	Energy emission and its penetration, ionization.

Safety regulations:

Some of the developed countries have formulated general safety regulations to minimize the risks associated with tissue culture laboratories.

Some of the general precautions for the safety of a tissue culture laboratory are listed here:

i. Strict adherence to recommendations of regulatory bodies.

ii. Periodical meetings and discussions of local safety committees.

iii. Regular monitoring of the laboratories.

iv. Periodical training of the personnel through seminars and workshops.

v. Print and make the standard operating procedures (SOPs) available to all staff.

vi. Good record keeping.

vii. Limited access to the laboratory (only for the trained personnel and selected visitors).

viii. Appropriate waste disposal system for biohazards, radioactive wastes, toxins and corrosives.

Biohazards:

The accidents or the risks associated with the biological materials are regarded as biohazards or biological hazards. There are two main systems that contribute to the occurrence of biohazards (Table 33.6).

Table 33.6 Sources that contribute to biohazards

Biological material(s)
Tissue samples and cultures with human pathogens.
Human cells infected with viruses (including retroviruses)
Cells subjected to various genetic manipulations.
Operating processes
Preparation of the media.
Development of primary cultures, cell lines and other laboratory works.

1. The direct sources of the biological materials.

2. The processes or operations involved in their handling.

Control of biohazards:

Biohazards can be controlled to a large extent by strict adherence to the regulatory guidelines and maintenance programmes. Some important aspects are listed.

i. Microbiological safety cabinet or biohazard wood with pathogen trap filters have been developed.

ii. Vertical laminar-flow hood (instead of horizontal laminar-flow hood) is recently in use. This minimizes the direct exposure of the operator to the samples/processes.

iii. Pathogen containing samples are treated in separate rooms with separate facilities (centrifuge, incubator, cell counting etc.).

iv. Sterilization of all wastes, solid glassware etc. and their proper disposal.

v. Facilities for change of clothing while entering and leaving the rooms.

vi. Strict adherence to the access of designated personnel to the culture rooms.

USE OF THE HORIZONTAL LAMINAR-FLOW

Laminar-flow cabinets (hoods) are physical containment devices that act as primary barriers either to protect the material being manipulated within the hood from worker generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood. Cell culture applications utilize two types of laminar-flow hoods:

(a) The horizontal-flow clean bench (described here) and

(b) The biological safety cabinet (see Alternate Protocol). Both types of hoods use a high-efficiency particulate air (HEPA) filter and blowers that generate a non mixing stream of air. The horizontal laminar-flow clean bench is used to provide a near-sterile environment for the clean (i.e., non contaminating) handling of nonhazardous material such as sterile media or equipment. Because the air stream pattern directs the flow of air within the hood directly back to the hood operator and the room. Horizontal flow hoods are never to be used with infectious agents or toxic chemicals.

Materials

- 70% ethanol or other disinfectant
- Horizontal laminar-flow hood, certified for use
- Swabs (e.g., cheesecloth, paper towels)
- Pilot light–activated Bunsen burner (e.g., Touch-o-Matic, VWR)

Procedure

1. Completely clear the bench of the laminar-flow hood and disinfect the bench working surface and the left and right sides of the hood with 70% ethanol or other disinfectant. Do not spray the back (gridded) wall where the HEPA filter is housed
2. Turn the hood blower and lights on and let the air circulate within the hood 10 min before use.
3. Place items needed for the specific procedure into the hood, wiping each item with 70% ethanol or other disinfectant just before introducing it into the laminar environment.
4. Wash hands well before working in the hood and wear a clean laboratory coat and surgical gloves to further protect the work from shedding of skin flora that can contaminant any product
5. While working in the hood, perform all work at least 4 in. back from the front opening, and avoid rapid movements that might disrupt the laminar air flow. Avoid moving materials or hands in and out of the cabinet as much as possible.
6. If flame sterilization is needed in the hood for a particular application, use a burner that can be activated by a pilot light when needed, rather than one that burns constantly.
7. When work is completed, remove all material from the laminar work bench, clean any spills, and disinfect the bench working surface by wiping with 70% ethanol or other disinfectant.
8. 8. Turn off hood blower and lights.

USE OF THE VERTICAL LAMINAR-FLOW BIOSAFETY CABINET

Biological safety cabinets provide a clean, safe environment for both the worker and the product.

Materials

- Class II, Type A Biosafety Cabinet (BSC), certified for use
- Pilot light–activated Bunsen burner (e.g., Touch-o-Matic, VWR) or electronic
- Incinerator (e.g., Bacti-Cinerator III, VWR)

Closed-front laboratory gowns (for personnel working with biological agents)

1. Turn the hood blower on and verify air flow by feeling (by hand) the current near the front grill of the work surface. Turn the germicidal UV light off if it is on. Turn the fluorescent light on.

2. Wash and gown as required for the operation (see Basic Protocol 1, steps 1 to 5).

3. Wipe down the entire interior cabinet work surface area with 70% ethanol or other appropriate disinfectant

4. Let blower run for 10 min to filter the cabinet air of any particulates.

5. Raise the front view window as needed to bring necessary items into the cabinet. Wipe each item with 70% ethanol or other disinfectant as it is placed in the cabinet.

6. Organize the work surface for a clean-to-dirty work flow. Place clean pipets, flasks, and sterile media bottles at one side of the cabinet; place discard pans, spent cultures, and other wastes on the other side.

7. Return the view window to the 8-in. operating level. Wait ~10 min for the blowers to filter the disturbed cabinet air before starting work.

8. While working, keep all material and perform work ≥4 in. back from the front opening of the cabinet, and minimize rapid movements or activity.

Keep the view window opening as close to 8 in. as allows reasonable access to the work surface and equipment

9. If direct flame sterilization of items within the cabinet is necessary, use an electric burner or pilot light–activated flame burner located at the back of the work space.

10. At the end of the procedure, enclose all contaminated materials. Clean the cabinet work surface with 70% ethanol or other disinfectant, being especially careful to wipe any spills of culture suspensions or media that can serve as future contamination points. Clear all material from the cabinet.

11. Let the blower run for ≥10 min with no activity to remove any aerosols that were generated. During this period, turn off the fluorescent light and turn on the germicidal UV light. Allow the UV light to operate ≥30 min.

CO_2 INCUBATOR

Inside a CO_2 incubator, also known as a gassed incubator, an atmosphere is created that is as natural as possible to develop cell and tissue cultures. This way of cultivating living organisms is called in vitro and is the main application for CO_2 incubators.

The process can take several weeks, during which sample growth and safety are always the top priority. To guarantee these aspects, the temperature, humidity, and CO_2 content must match the cell culture requirements as precisely as possible.

Structure

A CO2 incubator has an interior which is completely sealed off from the environment to ensure the atmosphere inside the unit cannot be affected by

external factors. To enable laboratory technicians to still view the samples during the growth phase, most CO2 incubators feature a glass door as well as the regular door, which provides additional protection against contamination.

The interior is made of rust-resistant materials such as stainless steel and should have as few sharp edges and grooves as possible to avoid any places where contamination could be hidden. In the sensitive field of cell cultivation in particular, just one single germ can ruin weeks of work.

The air coming in flows through a sterile filter and must be distributed evenly throughout the whole interior, as the samples are usually positioned on shelves at different heights. The challenge lies in creating homogeneity through the entire interior, to ensure a uniform supply of CO2 for all samples at constant climate and humidity conditions.

Applications of CO_2 incubators in the medical, pharmaceutical, and other related industries

Application

CO2 incubators are most frequently used in medical research and the pharmaceutical industry. However, they also provide sterile conditions for cultivation in other fields where cells must grow in a completely germ-free environment.

1. CO_2 incubators for tissue engineered products

Tissue engineered products are medical products specifically developed for novel treatments. With these new types of treatment, scientists hope to cure previously incurable diseases such as serious malignant tumors or

cardiovascular failure. CO_2 incubators are used to cultivate cell and tissue cultures in the research, development, and quality assurance of these products.

- As part of research into lipid-based gene therapy on a human skin model, BINDER CO_2 incubators were used to isolate epidermal keratocytes, in neutral red tests, and to prepare epidermal keratinocytes for transfection.
- Cells for cell therapy are produced in authorized centers. They are processed by CO_2 incubators in class C clean rooms.
- The Swiss Center for Regenerative Medicine (SCRM) at the University of Zurich uses CO_2 incubators to research therapies based on tissue engineering, i.e., the cultivation of tissue and cell clusters. This enables living tissue such as heart valves and blood vessels to be grown from stem cells in the laboratory.

2. CO_2 incubators for in vitro fertilization

In vitro fertilization (IVF) describes a method of artificial fertilization used in human reproductive medicine. This application aims to fuse an egg and sperm cell together in a petri dish along with a cell culture medium.

- The human embryos then start to fertilize and develop in a CO_2 incubator, before being transplanted into the uterus after two to three days. Optimal conditions in the incubator chamber are approximately 37°C, 5 or 6 vol. % CO2, and humidity of around 95%.

- Staff labels the petri dishes very precisely to avoid mix-ups. The inner glass doors of the CO_2 incubators mean the dishes can be monitored at all times too.

- The process is similar in veterinary medicine, for example with cattle. The oocytes are put into a petri dish together with the sperm and, during an incubation period of 21 hours, the sperm fertilize them. On the eighth day after fertilization, the embryos are transferred from the CO_2 incubator into the recipient animals.

3. CO_2 incubators in diagnostics

The study of cell cultures plays a crucial role in the diagnostic analysis of pathogens. The results obtained from such investigations enable precise proof of hygiene levels to be formulated, and the degree of biopharmaceutical resistance to viruses to be evaluated. One method often used in this context is the swipe sample, where cell cultures which are susceptible to viruses are exposed to them in order to test their biological function.

CO_2 incubators are the right tool for the various process steps involved, such as thawing and transferring the cells, as well as infecting the cell lines and dyeing the cell cultures. During this application, conditions in the interior are usually set to 37°C and 5 vol. % CO_2.

CO_2 incubators are particularly good for diagnostic purposes or virus identification, since they maintain extremely stable incubation conditions. Inner glass doors for segmented access help here too. What's more, the risks of cross-contamination and the silent spread of contamination are reliably eliminated at all times thanks to automatic hot-air sterilization at 180°C.

4. CO_2 for developing biosensors

Biosensors are cropping up more and more in fields such as medicine, fermentation monitoring, food quality control, and environmental analysis. A biosensor converts a biologically sensitive element into a digital signal, which

in turn can be used to perform analyses by means of physical or chemical stimulation.

Possible biologically sensitive elements:

- Enzymes
- Antibodies
- Organelles
- Whole cells
- Networks of cells

5. CO_2 incubators in cancer research

CO_2 incubators play an important role in preparing samples and tests in all areas of cancer research such as drug research and the development of 3D invasions, assays, and biosensors – from a simple monolayer through to the reproduction of patient tumors by means of 3D cell culture models (drug research and development of treatments).

In the fields of immunology and tumor biology (how malignant tumors develop), CO_2 incubators are used at 37°C, 5 vol. % CO_2, and 95% relative humidity under normoxic conditions, as well as CO2/O2 incubators at 37°C, 5 vol. % CO_2, and hypoxic 1 vol. % O_2, depending on the cell cultures involved.

In clinical research, for example, that into oncogenes and tumor suppressors, CO_2 incubators is used at 37°C and 5 vol. % CO_2 under normoxic conditions. The American Association for Cancer Research AACR's mission is to prevent and cure cancer through research, continuing education, communication, and cooperation.

Cryogenic preservation (storage below –130°C) of cell cultures is widely used to maintain reserves of cell cultures. Besides providing a valuable back-

up supply, properly stored cultures also reduce alterations in or loss of culture characteristics. This “culture drift” results from the phenotypic and genotypic changes that accumulate over time in most cell lines as they age and evolve in culture.

The success of the freezing process depends on four critical areas:

1. Proper handling and gentle harvesting of the cultures

2. Use of appropriate cryoprotective agents

3. A controlled rate of freezing

4. Storage at cryogenic temperatures.

Nonsterile

• Marking pen suitable for labeling plastic cryogenic vials

• Controlled-rate freezing unit

• Hemocytometer

Sterile

• Complete cell culture medium containing serum or any other supplements necessary for the culture being frozen.

• Pipettes .

• 15-mL screw-capped centrifuge tubes .

Calcium- and magnesium-free PBS (CMF-PBS) for rinsing the cultures.

• Trypsin or other appropriate dissociating solution • 2-mL screw-capped plastic vials specially designed for cryogenic use .

• Cryoprotective medium: complete culture medium containing 5% (v/v) dimethyl sulfoxide (DMSO) or 10% (v/v) glycerol.

• Trypan blue solution or other vital staining solution for determining the viability of the cells during counting.

Procedure

1. **Culture selection and examination**:

Prior to freezing, the culture should be maintained in an actively growing state (log phase or exponential growth) to ensure optimum health and good recovery. Ideally, the culture medium should be changed 24 hours prior to harvesting. It is also recommended that the culture be tested for the presence of microbial contaminants, especially mycoplasma, and have its identity (species and cell type) confirmed by appropriate methods. Using an inverted-phase contrast microscope, check the general appearance of your culture. Look for signs of microbial contamination. It is also important to examine the culture with the unaided eye to look for small, isolated fungal colonies that may be floating at the culture medium-air interface and thus are not easily detected with the microscope. If antibiotics have been used for growing your cultures, then maintaining the cultures antibiotic-free for at least one to two weeks prior to freezing will make it easier to uncover any cryptic culture contaminants.

2. Cell harvesting: Your standard protocol routinely used for sub culturing your cell cultures can generally be used. Be as gentle as possible during harvesting since it is very difficult for cells damaged during harvesting to survive the additional damage that occurs during the freezing and thawing processes. The amounts of reagents recommended in this procedure are for a

75-cm2 (T-75) flask; volumes should be adjusted accordingly for other culture vessel sizes.

2a. Using a sterile pipette, remove and discard the culture medium. Any materials and solutions coming into contact with cells should always be disposed of properly.

2b. Rinse the cell monolayer with 5 to 10 mL of CMF-PBS to remove all traces of fetal bovine serum.

2c. Add 3 to 5 mL of trypsin (in CMF-PBS) or other appropriate dissociating agent to the and incubate at 37°C. Prewarming the enzyme solution will usually shorten the exposure period.

2d. Check the progress of the enzyme treatment every few minutes on an inverted phase-contrast microscope. Once the cells have rounded up, gentle tapping of the flask should detach them from the plastic surface. Add 5 mL of serum-containing growth medium to inactivate the trypsin solution. Vigorous pipetting may be required to wash off any remaining cells from the bottom of the culture vessel or to break up cell clumps into a single cell suspension. The removal or inactivation of the dissociating agent is critical for optimum recovery of frozen cells. If the dissociating agent cannot be directly inactivated, then it will be removed when the cells are centrifuged in the next step (see below). 2e. Collect the suspended cells in a 15-mL centrifuge tube. Remove and set aside a small sample for cell counting and then spin the remaining cell suspension at approximately 100 x g for 5 minutes to obtain a soft cell pellet. Count the cells using a hemocytometer while the tube is spinning. Use the trypan blue solution to check their viability

3. Cryoprotective agents: Cryoprotective agents are necessary to minimize the damage that occurs to cells during the freezing process. A wide variety of chemicals have been used to provide cryoprotection, including polyvinyl-pyrrolidone, ethylene glycol, methanol, and methyl acetamide. However, the

most common cryoprotectants are dimethylsulfoxide (DMSO) and glycerol. DMSO (ATCC® cat. no. 4-X, 5 x 5 mL) is most often used at a concentration of 5 to 10% (v/v) in freezing media; the optimum concentration varies with the cell line.

Glycerol is most often used in freezing media at a final concentration of 5 to 15%; again, the optimum concentration depends on the cell line. Increasing the serum concentration in the cryoprotective medium is often used to increase the survival rate of cells that are difficult to preserve. Serum concentrations as high as 90% to 95% (no medium, just serum plus the cryoprotective agent) are sometimes used, especially with sensitive hybridoma cell lines. For cells normally grown in serum-free medium, adding 50% conditioned medium (serumfree medium in which the cells were grown for 24 hours) to both the cell freezing and the recovery media may improve their post-freezing recovery and survival. The addition of 10% to 20% cell culture-grade albumin to serum-free freezing medium may also increase post-freezing survival.

4. Cell freezing: A slow and reproducible cooling rate is very important to ensure good recovery of cultures. A decrease of –1 to –3°C per minute will usually work for most animal cell cultures. The best way to control the cooling process is to use a programmable electronic freezer unit. However, there are several commercially available mechanical freezer units that give a satisfactory and reproducible cooling rate when placed overnight in a –70°C to –90°C freezer

4a. Freeze the labeled vials in an appropriate controlled rate freezer unit according to the manufacturer's directions. If no commercial freezing unit is available, a homemade substitute can be constructed by placing the vials in a small box of polystyrene foam or insulated cardboard, which is then placed in a –70°C to –90°C freezer overnight. While this approach works with many cell lines, it does not give controlled, uniform, or reproducible cooling and is not recommended for valuable or irreplaceable cultures. 4b. No matter which

cooling method is used, it is important that the transfer to the final storage location be done quickly and efficiently. If the transfer cannot be done immediately, the vials can be placed on dry ice for a short time. This will avoid damage to cultures by inadvertent temporary warming during the transfer process. Warming during this transfer process is a major cause of variation in culture viability upon thawing.

5. Cryogenic storage: Always keep the storage temperature below –130°C for optimum survival. Cells may survive storage at higher temperatures but viability will usually decrease over time. The ideal storage container is a liquid nitrogen freezer where the cultures are either stored submerged in the liquid nitrogen or suspended in the vapor phase above the liquid nitrogen. For safety reasons (#6 below) storage in vapor phase is preferred. There are several important points to remember for maintaining a liquid nitrogen-based cell repository:

5a. Make sure that the labeling system is suitable for (permanent) cryogenic storage.
5b. Keep good records of the locations of the frozen cultures and a full history of their characteristics and growth and handling requirements. Remember that the frozen cultures may be around longer than the person who froze them.
5c. Be prepared for emergencies! Liquid nitrogen freezers can fail. There should be a person responsible for frequent checks (preferably daily or at least weekly) on the status of a liquid nitrogen freezer. There are a variety of commercially available alarm systems that can be used to continually monitor their status. Cultures that are very valuable or irreplaceable should be stored in at least two separate locations. ATCC provides a safe deposit service for this purpose; contact ATCC Professional Services at (703) 365- 2700 for additional information on this service.
6. Culture recovery: Special care should be taken when thawing cultures that have been stored submerged in liquid nitrogen instead of in the vapor phase

above liquid nitrogen. If a vial leaks during submerged storage it will slowly fill with liquid nitrogen; upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Refrigerated centrifuge works on the concept of sedimentation principle by holding up the sample tubes with a capacity of 2ml, 10ml and 50ml in rotation around a fixed axis. In this, the centripetal force causes the denser substances to separate out along the radial direction in the bottom of the **centrifuge** tube.

Refrigerated Centrifuge

Refrigerated laboratory **centrifuges** temperature ranges as wide as -20C – 40C, making them perfect for DNA, RNA, PCR or antibody analysis. A **refrigerated** laboratory **centrifuge** can obtain rotational speeds of over 30,000 rpm, and a relative **centrifugal** force (RCF) of over 65,000 x g.

PROCEDURE:

- Wear disposable gloves when there is a risk of contamination.
- Ensure that the centrifuge is switched on at the wall plug socket.
- Keep the lid of the centrifuge open when it is not in use
- Unscrew the lid of the rotator bucket to be used.
- Place the tubes in the tri union rings inside the rotator buckets.
- 8. Ensure that the centrifuge is balanced, making sure on opposite sides there is an identical tube balanced by weight.
- Ensure that the rotator buckets are firmly closed with the covers in place for each centrifugal run.
- 10 Set the time and speed on the centrifuge according to study protocol and press start.

SAFE OPERATING PROCEDURE

- If the refrigeration unit is operating, keep the chamber door closed as much as possible to prevent moisture build up inside the chamber.
- If you notice any unusual vibrations, sounds or odours, TURN THE POWER OFF until the cause can be determined.
- Turn the POWER switch OFF when centrifuge is not in regular use, to conserve power, and reduce equipment wear and tear.
- Keep the rotor and drive hub contact surfaces clean and lightly lubricated with 'Spinkote' lubricant, to prevent the rotor sticking to the drive-shaft.
- Ensure the rotor is securely installed prior to use.
- When removing the rotor, lift it vertically to avoid bending the driveshaft.
- Periodically defrost the chamber and wipe up accumulated moisture.
- Clean the condenser fins at the back of the instrument every 4-6 months.
- Do not operate the instrument if any panels have been removed – this is an electrical shock / mechanical injury hazard.
- Do not operate the equipment if the ambient temperature exceeds 38°C.
- Do not operate the equipment unless it has a valid electrical testing tag.
- Appropriate personal protective equipment like respirators, face shield, goggles and gloves shall be used when required.
- Risk assessment must be conducted to decide on the type of PPE required for the work.
- ***Procedure***

FREEZER 80°C

The freezer is intended for ensuring an environment for material that requires preservation at temperatures below zero. Optimal operation of the freezer is achieved through proper installation and maintenance (control of temperature, defrosting and cleaning).

PROCEDURES

- Freezer must have proper electrical isolation, and must be installed according to Thermo scientific Manufacturer's Manual.
- Ensure good ventilation to allow outward flowing air to escape and to prevent condensation of moisture on the outside of the freezer.
- Do not install freezer close to heat sources.
- Ensure that freezer is placed on a level surface.
- To prevent overloading the outlet, do not connect the fridge directly to a power supply supplying other equipment.
- Check temperatures daily and record in the appropriate Logbook

Defrosting and Cleaning

- Defrost freezers every six months, or more frequently if required, specifically in the event of leakage of biological materials onto the internal surface of the freezer or when the ice build-up on the internal wall reaches 5-6 mm in thickness whichever comes first.
- Ensure that materials kept in the freezer are clearly marked.
- Identify temperature sensitive reagents/solutions which need to be relocated during defrosting.
- Consider safety issues for the temporary space used for biological materials (mycobacterial cultures, specimens etc) while defrosting.
- Identify adequate volume of available space in another freezer.
- Notify staff of contents of relocation.
- Note location of relocated contents in an appropriate freezer logbook.
- Disconnect the plug from the main socket. Open the door and leave it open.
- To accelerate the process put a pan with warm (not boiling) water into the compartment.
- Place another container under the drain hole to catch the defrosted water.
- Sponge up any defrosted water.

- Once all the ice has been removed, wash with a sponge and cleaning solution.
- Dry the internal compartment using paper towels/soft cloth.
- Never use a sharp object to chip the ice during the defrosting process.
- Never use abrasives.
- Dry with a soft cloth.
- Connect the freezer to the main power supply and switch on.
- Relocate the freezer contents once the required temperature has been reached.
- Do not over pack.
- Notify staff of contents of relocation.
- Clean the condenser every three months with a brush or vacuum cleaner.

Servicing

- Check the compressors for unusual sound or overheating. Issue an Equipment Failure Report and alert the technical service for maintenance, even if the equipment is still performing.

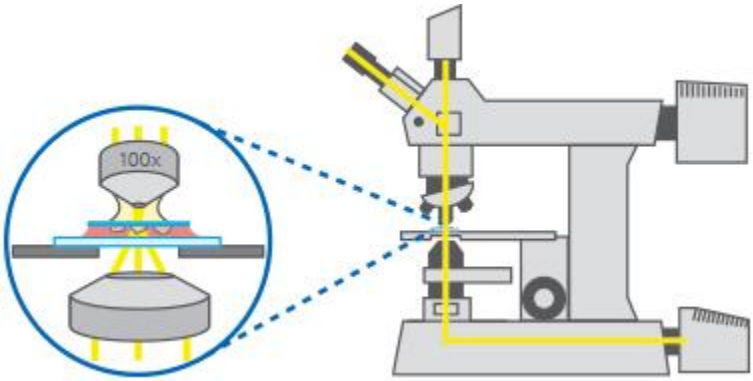
INVERTED MICROSCOPE

An **inverted microscope** is a **microscope** with its light source and condenser on the top, above the stage pointing down, while the objectives and turret are below the stage pointing up. It was invented in 1850 by J. Lawrence Smith, a faculty member of Tulane University (then named the Medical College of Louisiana).

I. Inverted microscopes are constructed with the tip of the objective pointing upward so as to view the specimen from below. The objective is underneath the stage and light is directed on the specimen from above. This type of **microscope** is suitable for viewing culture vessels such as Petri dishes.

Inverted microscopes are popular for live cell imaging, because:

- Cells sink to the bottom and onto the cover slip for adherence
- Sample access from the top (e.g., for liquid exchange or micropipettes)
- No contact between objective and sample—sterile working conditions are possible



APPLICATIONS:

Inverted microscopy is a very popular technique for live cell imaging. Here, living cells are observed through the bottom of a cell culture vessel. This technique has several advantages over upright microscopy.

Most cells naturally sink to the bottom of the vessel and—if no suspension cells are used—adhere to the surface, meaning that they are spread across one focal plane. When growing in vessels that are suitable for inverted microscopy, cells have access to larger amounts of medium than if they were squeezed between a cover slip and a slide. In addition, sample access from the top is possible, for medium exchange or micropipettes, for example. Another very important advantage is sterility: as there is no contact between the objective and the sample, sterile working conditions are guaranteed.

Principle:

In an inverted microscope, the source for transmitted light and the condenser are placed on the top of the stage, pointing down toward the stage. The objectives are located below the stage pointing up. The cells are observed through the bottom of the cell culture vessel. To meet the criteria for successful inverted microscopy, the bottom of the culture vessel must have the highest optical features, which are given for the ibidi Polymer Coverslip and the ibidi Glass Coverslip.

Inverse microscope

The optical microscopes can be sorted on a structure basis accordingly to the purpose of their use; they can be upright or inverted.

Principle

While in the inverted microscope the objective is located under the specimen and the condenser above, in the upright microscope (the most used microscope) the objective is located above the specimen and the condenser below.
In 1850 J.Lawrence Smith invented the inverse microscope that was shown, for the first time, to the scientific community during the World's Fair in 1852. This invention was extremely important for the evolution of the microscopy has allowed, for the first time in history, to observe living and large specimens as until then all the specimens should be small and preserved on a slide.

Structure

As said before, when comparing the structure of the upright microscope with the structure of the inverse microscope the main differences between them are the location of the objective and condenser. In this kind of instrument the

lenses are located under a transparent viewing stage. The stage is fixed and the lenses move in a vertical axis, this movement permits to focus the sample from a further or closer distance. The illumination source is located above the subject and the direction of the light rays (which are convey from above through the objective), are modified by a group of mirrors making the light reach the eye from below. The inverse microscope has the possibility to fit video cameras, fluorescence illumination, material to confocal scanning and other applications suitable to diverse microscope techniques.

Applications

This kind of microscope is manly used to observe living cells and organisms big enough to fit a large container, which allows the specimen to be under natural conditions while in other types of microscopes the sample is under glass slide. As this microscope allows the direct manipulation of the subject (as it has space above the sample to manipulate the micro tools used) it is widely used in micromanipulation applications. In metallurgical applications where polished samples can be placed on top of the stage and viewed from underneath using reflecting objectives.

Advantages

- Possibility to observe living and large specimens
- Observe the specimen for a longer period of time
- Ability to manipulate directly the sample

Disadvantages

- It is an expensive microscope

HEMOCYTOMETER

A **hemocytometer** is a specialized slide which is used for counting cells. It is actually a glass slide which has a 3×3 grid etched into it. Carved in it are intricate, laser-etched lines that form a grid. It also has its own coverslip

Every person having anything to do with microbiology, biotechnology, pathology or other related fields will be required to know their way around a hemocytometer. Used for the count of various micro-particles or microorganisms, a hemocytometer is a special slide, and much more expensive than your average glass slide! It can be used to count the number of red blood cells in a sample, as well as white blood cells, microbes like yeast, and many others.

A hemocytometer looks, from a distance, like an average glass slide, just heavier. However, it is much more than that. Carved in it are intricate, laser-etched lines that form a grid. It also has its own coverslip, which is different from a regular coverslip. On the slide, there are marked grooves that appear like an 'H'. The horizontal line of the 'H' separates the 2 grids for counting. Therefore, each slide has two identical grids for counting cells. The depth of these 2 grids is a fixed 0.1mm

Hemocytometer calculation

Each grid is a square with the dimensions of 3×3 mm^2. This square has three equidistant vertical and horizontal lines. These divide it into 9 smaller squares of 1×1 mm^2 each. These are separated from each other by triple-ruled lines. Of these 9 squares, the 4 corner squares are used for the counting of bigger cells, like WBCs, while the center square is used to count smaller cells, such as RBCs.

The 4 corner squares of the main grid are further divided into 16 smaller cells. The centre square of the main grid is divided into 25 smaller squares, each of which is again divided into 16 smaller squares. The sample to be

counted is loaded onto the slide *after* the coverslip has been placed. Excess fluid drains into the grooves on the side. However, the person loading the sample must be extremely careful while loading.

Counting grid

This covers the structure and design of the hemocytometer, but to understand how counting and calculation is done, let's considers the example of counting WBCs for the corner squares, and RBCs for the center square.

Hemocytometer counting

RBCs, being smaller in size and larger in number, are counted in the center square. This has a greater number of divisions and therefore makes counting easier. As mentioned above, the center square contains 25 smaller squares. The area of each of these is 1/25 mm^2, which is 0.04 mm^2. Once the sample is loaded, not all the cells are counted. Out of 25, any 5 squares are picked for the counting. The division of each of these 0.04 mm^2 squares into 16 smaller ones makes it easier for the person to count the number of cells, rather than just having to count in an empty square. There are a number of patterns to select the 5 squares that should be counted. The corner 4 and center square can be picked, or any of the diagonal lines of squares.

Centre square of hemocytometer

Once the number of cells in 5 squares has been counted, their mean is taken. Let '*n*' be the mean. Therefore, the average number of cells in each of the tiny 0.04 mm^2 squares is *n*. The volume of each of these cells is 0.04 x 0.1 = 0.004 mm^3. The number of cells in 1 mm^3 is $n/0.004$. Therefore, the total number of cells in 1ml is $(n/0.004)$ x 1000. We multiply by one thousand as 1000 mm^3 = 1 cm^3; and 1 cm^3 = 1 mL

Counting pattern for the center square

WBC Count

When WBCs are counted, the calculation is much easier. WBCs are counted in the 4 corner squares of the main grid. These squares have an area

of 1 mm^2 each. To get the WBC count, the number of cells in each square is counted, and their mean is then calculated. Let the mean be '*n*'. The volume of each square is 1 x 0.1 = 0.1 mm^3. The number of cells in 1 mm^3 is $n/0.1$. Therefore, the total number of cells in 1ml is $(n/0.1)$ x 1000. We multiply by one thousand as 1000 mm^3 = 1 cm^3; and 1 cm^3 = 1 mL

Rules to be followed

While counting cells, certain things require attention. Some cells may not lie either inside or outside the square. Rather, they may fall on the border. Therefore, a simple practice of including cells that fall on the top and left border and excluding cells that fall on the bottom and right border is followed. However, this is not a rule. The basic principle is that any 2 adjacent borders should be counted, and the remaining 2 borders should be rejected. Also, this selection criteria must apply to all the squares being counted.

Border rule

Sometimes the solution of the sample may be too concentrated. If it is too highly concentrated, the cells will overlap and thus the counting will be wrong. Therefore, such concentrated cell solutions need to be diluted with an appropriate solution. This dilution also needs to be factored into the calculations. For instance, if the sample has been diluted by 10x, the final answer obtained from the calculations must be multiplied by 10.

pH METER

pH is a measurable parameter between the values of 0 and 14, provided the concentration of the solution does not exceed 1M. Solutions with a pH<7 are acidic, whereas those with a pH>7 are alkaline. A pH meter is a device that measures the changes in the activity of hydrogen ions in solution.

What are acids and alkalis?

According to the Arrhenius definition, acid is any substance that produces dissociated hydrogen (H^+) ions or hydronium ions H_3O^+ when it is

dissolved in water. A base is a substance that produces dissociated hydroxyl (OH^-) ions when it is dissolved in water.

The concept of pH was proposed by the Danish chemist Søren Peder Lauritz Sørensen in 1909. The pH quantifies the H^+ ions in a solution. The notional definition of pH is the logarithmic of the reciprocal of hydrogen ion activity, a_{H^+}, in a solution:

$$\mathrm{pH} = -\log_{10}(a_{\mathrm{H}^+}) = \log_{10}\left(\frac{1}{a_{\mathrm{H}^+}}\right)$$

Equation 1. *The notational definition of pH, expressed in mathematical terms.*

The pH is directly related to the proton concentration only when the solution is very dilute. However, pH is accurately determined by pH-sensitive, ion-selective electrodes which respond to proton *activity*, rather than changes in proton concentration. The notional definition of pH is therefore discarded and ideally defined according to the Nernst equation, which for the H^+ ion can be expressed as follows:

$$E = E' + \frac{RT}{F}\ln(\alpha_{\mathrm{H}^+}) = E' - \frac{2.303RT}{F}\mathrm{pH}$$

Equation 2. The operational expression of pH. The electrode potential (E) of a pH-sensitive electrode follows the Nernst equation as shown. The definition of the parameters is included in the text.

E is the measured potential, E' is a standard electrode potential (i.e., the constant value of the electrode potential for a_{H^+} = 1, and the specific

electrode/solution conditions for each case), *R* is the gas constant, *T* is the temperature in Kelvin, *F* is the Faraday constant. The pH-sensitive, ion-selective electrode is a linear function of the pH when the pH is defined as a measure of proton *activity*.

The standard methodology for measuring pH

pH meters consist of a glass electrode made of a speciality glass membrane that is sealed at the end forming a bulb. Inside the glass is an internal standard acidity solution, usually 0.1 M HCl, along with an internal reference electrode, RE_{in} (typically an Ag/AgCl wire electrode). This solution is called the reference solution of known pH, 7.

A second electrode RE_{ext} is placed in an external tube immersed in KCl. This external tube forms a concentric enclosure around the first enclosed glass tube containing the 0.1 M HCl (the internal tube).

The external tube is made of pH sensitive glass and is in contact with the test solution through an opening called a porous diaphragm. The inclusion of a reference electrode encased by the internal tube is necessary as its pH is known and can be compared to a test solution so that its pH value can be determined. This set-up is called a combination pH electrode.

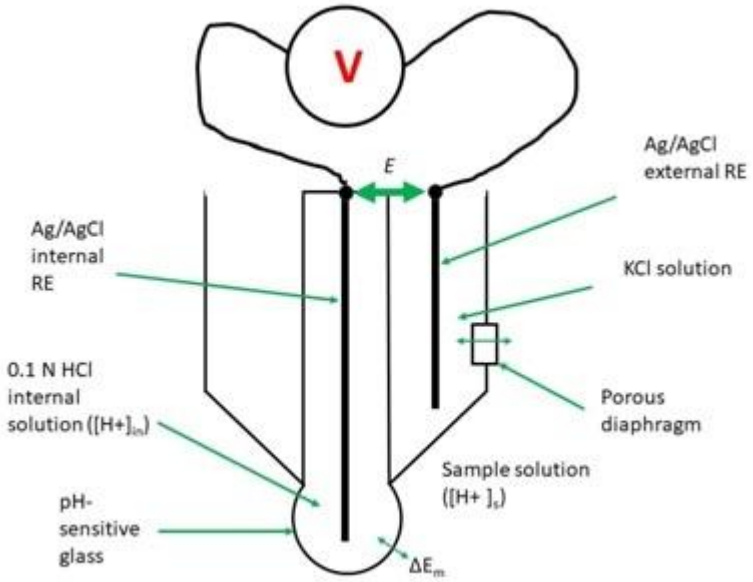


Figure 1. *A combination pH electrode. All components are shown and referred to in the text. RE refers to Reference Electrode.*

Operation of pH meter

The pH meter operates like a voltmeter. The pair of electrodes in the combinatorial set-up can measure small changes in voltage (also called potential difference) in the order of millivolts. Changes in potentials are caused by the loss of electrons that correspond with the loss of H^+.

The voltage produced by the test solution is measured and compared with the voltage produced by the reference solution which is exposed to the test solution via the porous diaphragm. The difference in the voltage between the two is used to calculate the pH:

$$E = E_{\text{const}} - \Delta E_{\text{m}}$$

E_{const} refers to the potential difference of the reference electrode and DE_m is the change in the potential difference of the external glass membrane caused by the external test solution and internal KCl solution. DE_m can be calculated using the Nernst Equation:

$$\Delta E_m = 2.303 \frac{RT}{F} \log \frac{[H^+]_{in}}{[H^+]_s}$$

Calibration

The pH meter should be calibrated before each measurement using at least two standard buffer solutions of known pH values (typically around 4 and 7).

Magnetic stirrer

Magnetic stirrers use a rotating magnetic field to move a stir bar around in liquid samples. The movement of this stir bar mixes the sample thoroughly with rapid movement and agitation. The speed of the magnetic field is controlled by the user, so it can be customized to the specific sample that's being stirred. These stirrers should be used with glass or other non-metal beakers to prevent interference with the magnetic field.

Depending on the application, you can configure your magnetic stirrer with hot plates to heat your samples as they're stirred. With the use of heating plates, you can mix a wide variety of samples for different applications. *Magnetic stirrer* makes use the principle of magnetic field and vortex, put the stirred liquid in bottle on *magnetic stirrer* base, there will generate a rotating magnetic field around the base to drive stirrers circulating in a circle, then forming a vortex in the vessel, in this way to stir liquid.

At present, there are two types magnetic stirrers used in laboratory: *electric stirrer* and magnetic stirrer, among which magnetic stirrer is applicable for mixing low viscosity liquid or solid-liquid mixture.

Comparing to electric stirrer, magnetic stirrer has following merits:

Magnetic stirrer

1. Use high quality continuous current motor, low noise, stable speed regulation;
2. The stirrer is made from high quality magnetic steel covered PTFE, heat resistant, wear resistant and corrosion resistant;
3. Magnetic stirrer can be used in airtight vessel, very convenient;
4. Equipped with heating device, greatly convenient for application which requires heating and stirring.

What's the function of magnetic stirrer? In general, there are two *magnetic stirrer function* s: heating and stirring.

The stirring function of magnetic stirrer:

1. To uniformly mix the reaction material and balance temperature distribution, in order to speed up reaction or evaporation, then shorten reaction time;
2. When heating material in an airtight vessel, magnetic stirring can prevent bumping.

The heating function of magnetic stirrer:

The temperature range of heating device generally is between room temperature to 100℃, for some simple type magnetic stirrer, it only has stirring function.

- A **magnetic stirrer** is equipment used to create rotating magnetic field. The magnetic stirrer is designed such that there is a small bar magnet and a stand or plate containing the rotating magnet.

- In general the bar magnet is coated with plastic and plate contains rotating magnet. It is possible to create a rotating magnetic field with the help of a rotating magnet.

Working of Magnetic Stirrer:

- An **external magnetic field** is applied to the magnetic stirrers mix solution which facilitates the rotating of the small magnetic bar placed in the mixture of interest.
- The way this works is the rapidly rotating magnetic field makes the bar magnet rotate which ends up stirring the liquid. Since their size is usually small they are highly preferred in laboratory experiments.

Functions and uses of a Magnetic Stirrer:

- The magnetic stirrers are also known as **magnetic stir plates** and quite commonly used for experiments in chemistry and biology.
- There are very useful when you need to mix component, either solids or liquids and get a homogeneous liquid mixture. Some of the common samples include bacterial growth media as well as buffer solutions.
- The main function of a stirrer is to agitate the liquid for speeding up the reactions or improving mixtures. A magnetic stirrer is often used with hot plates.

Types of a Magnetic Stirrer:

There are several types of **Magnetic Stirrer** available and it all depends on your selection of size, application and configuration. The various different type are listed below:

Magnetic Mini Stirrer:

As the name suggests, the **mini magnetic stirrer** is a compact sized stirrer that occupies very little space and allows the users to regulate the speed with great precision with the help of the controls provided. It has been wisely designed to be resistive to harmful chemicals present in a laboratory environment. In addition to which it also consists of a speed regulator that ensures that the device never exceeds the maximum speed.

Magnetic Stirrer with Timer:

Again the name says it all. This is the type of stirrer that has the ability to automatically shut off the motor after the set amount of time. The way this works is that it uses an in built timer to shut off the stirrer when the pre – selected period of time has been over. Besides which the speed is also automatically reduced and the load is automatically removed.

Heavy-Duty Magnetic Stirrer:

The **heavy duty magnetic stirrer** are true to their name. They have a greater chemical resistance and are highly durable, besides which they also have a high mixing capacity. Additionally they have an internal electronic control device that regulates the speed automatically with respect to the load. Thus the heavy duty magnetic stirrer makes for a perfect instrument for use in a laboratory or production environment.

Battery Powered Magnetic Stirrer:

The battery powered magnetic stirrer come in handy where there is no electric outlet. Their main application is in incubators. They consist of rubber feet and their speed can be controlled with the help of the speed control knobs provided. In general alkaline batteries can be used with these devices though rechargeable batteries should work just fine.

Air Operated Turbine Magnetic Stirrer:

If you have the requirement for stirring liquid up-to one liter than the air operated turbine separator is the ideal equipment. The major advantage being that it eliminates the hazards associated with sparking from electrical sources.

Benefits of using a Magnetic Stirrer:

One of the major **advantages of a magnetic stirrer** is that it minimizes the risk of contamination since only there is only an inert magnet bar that is

placed inside the sample (fluid). Additionally this can also be easily cleaned. A manual stirrer is not as consistent as the magnetic one and the magnetic one is also critical for reproducible mixing or mixing over long time scale. For example, protein dialysis requires multi hour or overnight sample mixing and is sensitive to bacterial contamination.

Specifications of using a Magnetic Stirrer:

- There are many different **types of Magnetic Stirrers** available.
- Here are the specifications of a Magnetic Stirrer from Neuation, called i Stir Uno.
- This one has no moving parts and is truly maintenance free.
- It has software driven gradual acceleration and an adjustable speed.
- It is microprocessor controlled and has the last run memory storing ability.

MICROPIPETTES

Objective: • Learn how to use micropipettes

Background: Micropipettors are the standard laboratory equipment used to measure and transfer small volumes of liquids. You will use them throughout this semester and in advanced courses that you take in the future. It is essential that you master their use if you are to be successful in your experiments. A. Parts of a micropipette a b c d f e a b c d f e a. Plunger button b. Tip ejector button c. Volume adjustment dial d. Digital volume indicator e. Shaft f. Attachment point for a disposable tip

B. Three sizes of micropipettes

The Micropipettors in this laboratory come in three different sizes each of which measures a different range of volumes. The three sizes are P20, P200 and P1000. These sizes are noted on the top of the plunger button. Size Micropipette

Range of volumes measured P20 0.5-20µl P200 20-200µl P1000 100-1000µl 0 22 C.

Adjusting Volume on micropipettes The black volume adjustment dial near the top of the micropipette allows you to adjust the volume that is measured. It can be dialed to the left or right to increase or decrease the volume.

The digital readout shows the volume that will be measured. As you turn the volume adjustment dial, the numbers in the digital readout will change.

On each of the three sizes of micropipettes (P20, P200, P1000) the digital readout has three numbers. These three numbers correspond to different volumes on the different size pipettes. See the figure below for instructions on interpreting digital readout.

In a P100, the top number refers to 1000's of µl, the middle number refers to 100'sµl and the bottom number refers to 10's of µl's

. In a P200, the top number refers to 100's of µl, the middle number refers to 10's µl and the bottom number refers to µ'ls.

In a P20, the top number refers to 10's of µl, the middle number refers to µl's and the bottom number refers to 1/10ths of µl.

D. Pipette Tips Liquids are never drawn directly into the shaft of the pipette. Instead, disposable plastic tips are are attached to the shaft. There are two sizes of tips. The larger blue tips are used for the P1000.

The smaller clear tips are used for the P20 and P200. The tips are racked in plastic boxes with covers. When you receive a box, it will be sterile. Please be careful when touching box or tips not to contaminate them. The box should be closed when not in use to prevent airborn contamination. **Inserting the Tip**

Select the correct size tips. Open the box without touching the tips with your hands.

Insert the micropipette shaft into the tip and press down firmly. This will attach the tip to the shaft. Remove the micropipettor with the tip attached. Close the box

without touching the tips with your hands. E. Punger Settings The plunger will stop at two different positions when it is depressed. The first of these stopping points is the point of initial resistance and is the level of depression that will result in the desired volume of solution being transferred. The second stopping point is when the plunger is depressed beyond the initial resistance until it is in contact with the body of the pipettor.

At this point, the plunger cannot be depressed further. This second stopping point is only used for the complete discharging of solutions from the plastic tip. F. Measuring and transferring a volume of liquid before measuring and transferring liquid:

- Choose the appropriate size micropipettor
- Adjust to the correct volume
- Insert tip on the shaft. Measuring and transferring liquid
- Depress the thumb knob to the first stop.
- Immerse the tip approximately 3 mm into the sample solution (step 1).
- Slowly release the thumb knob to the initial position (step 2). Watch as the solution is drawn up slowly into the tip. Do not release the plunger too quickly.
- Release might draw bubbles in the solution and might splash solution on the non-sterile shaft.
- Withdraw the tip from the sample solution.

UNIT II

CULTURE MEDIA FOR ANIMAL CELLS

The selection of an appropriate growth medium for the in vitro cultivation of cells is an important and essential step. The mammalian cells of an organ in the body receive nutrients from blood circulation.

For culturing these cells in vitro, it is expected that they should be provided with the components similar to those present in blood. In general, the choice of the medium mostly depends on the type of the cells to be cultured, and the purpose of the culture (growth, differentiation, and production of desired products). The culture media may be natural or artificial.

Natural Media:

In the early years, the natural media obtained from various biological sources were used.

Body fluids:

Plasma, serum, lymph, amniotic fluid, ascitic and pleural fluids, aqueous humour from eyes and insect hemolymph were in common use. These fluids were tested for sterility and toxicity before their utility.

Tissue extracts:

Among the tissue extracts, chick embryo extract was the most commonly employed. The extracts of liver, spleen, bone marrow and leucocytes were also used as culture media. Some workers still prefer natural media for organ culture.

Artificial Media:

The artificial media (containing partly defined components) have been in use for cell culture since 1950.

The minimal criteria needed for choosing a medium for animal cell cultures are listed below:

i. The medium should provide all the nutrients to the cells.

ii. Maintain the physiological pH around 7.0 with adequate buffering.

iii. The medium must be sterile, and isotonic to the cells.

The basis for the cell culture media was the balanced salt solution which was originally used to create a physiological pH and osmolarity required to maintain cells in vitro. For promoting growth and proliferation of cells, various constituents (glucose, amino acids, vitamins, growth factors, antibiotics etc.) were added, and several media developed.

Addition of serum to the various media is a common practice. However, some workers in recent years have started using serum-free media. The physicochemical properties of media required for tissue cultures are briefly described. This is followed by a brief account on balanced salt solutions, commonly used culture media and the serum-free media.

Physicochemical Properties of Culture Media:

The culture media is expected to possess certain physicochemical properties (pH, O_2, CO_2, buffering, osmolarity, viscosity, temperature etc.) to support good growth and proliferation of the cultured cells.

pH:

Most of the cells can grow at a pH in the range of 7.0-7.4, although there are slight variations depending on the type of cells (i.e. cell lines). The indicator phenol red is most commonly used for visible detection of pH of the media.

Its colouration at the different pH is shown below:

At pH 7.4 — Red

At pH 7.0 — Orange

At pH 6.5 — Yellow

At pH 7.8 — Purple

CO_2, bicarbonate and buffering:

Carbon dioxide in the medium is in a dissolved state, the concentration of which depends on the atmospheric CO_2 tension and temperature. CO_2 in the medium exists as carbonic acid (H_2CO_3), and bicarbonate (HCO^-_3) and H^+ ions as shown below.

$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^-_3$

As is evident from the above equation, the concentrations of CO_2, HCO^-_3 and pH are interrelated. By increasing the atmospheric CO_2, the pH will be reduced making the medium acidic.

Addition of sodium bicarbonate (as a component of bicarbonate buffer) neutralizes bicarbonate ions.

$NaHCO_3 \leftrightarrow Na^+ + HCO^-_3$

In fact, the commercially available media contain a recommended concentration of bicarbonate, and CO_2 tension for the required pH. In recent years HEPES (hydroxyethyl piperazine 2-sulfonic acid) buffer which is more efficient than bicarbonate buffer is being used in the culture media. However, bicarbonate buffer is preferred by most workers because of the low cost, less toxicity and nutritional benefit to the medium. This is in contrast to HEPES which is expensive, besides being toxic to the cells. The presence of pyruvate in the medium results in the increased endogenous production of CO_2 by the cells. This is advantageous since the dependence on the exogenous supply of CO_2 and HCO^-_3 will be less. In such a case, the buffering can be achieved by high concentration of amino acids.

Oxygen:

A great majority of cells in vivo are dependent on the O_2 supply for aerobic respiration. This is in fact made possible by a continuous supply of

O_2 to the tissues by hemoglobin. The cultured cells mostly rely on the dissolved O_2 in the medium which may be toxic at high concentration due to the generation of free radicals. Therefore, it is absolutely necessary to supply adequate quantities of O_2 so that the cellular requirements are met, avoiding toxic effects.

Some workers add free-radical scavengers (glutathione, mercaptoethanol) to nullify the toxicity. Addition of selenium to the medium is also advocated to reduce O_2 toxicity. This is because selenium is a cofactor for the synthesis of glutathione.

In general, the glycolysis occurring in cultured cells is more anaerobic when compared to in vivo cells. Since the depth of the culture medium influences the rate of O_2 diffusion, it is advisable to keep the depth of the medium in the range 2-5 mm.

Temperature:

In general, the optimal temperature for a given cell culture is dependent on the body temperature of the organism, serving as the source of the cells. Accordingly, for cells obtained from humans and warm blooded animals, the optimal temperature is 37°C.

In vitro cells cannot tolerate higher temperature and most of them die if the temperature goes beyond 40°C. It is therefore absolutely necessary to maintain a constant temperature (± 0.5°C) for reproducible results.

If the cells are obtained from birds, the optimal temperature is slightly higher (38.5°C) for culturing. For cold blooded animals (poikiltherms) that do not regulate their body heat (e.g. cold-water fish), the culture temperature may be in the range of 15-25°C. Besides directly influencing growth of cells, temperature also affects the solubility of CO_2 i.e. higher temperature enhances solubility.

Osmolality:

In general, the osmolality for most of the cultured cells (from different organisms) is in the range of 260-320 mosm/kg. This is comparable to the osmolality of human plasma (290 mosm/kg). Once an osmolality is selected for a culture medium, it should be maintained at that level (with an allowance of ± 10 mosm/kg). Whenever there is an addition of acids, bases, drugs etc. to the medium, the osmolality gets affected. The instrument osmometer is employed for measuring osmolalities in the laboratory.

Balanced Salt Solutions:

The balanced salt solutions (BSS) are primarily composed of inorganic salts. Sometimes, sodium bicarbonate, glucose and HEPES buffer may also be added to BSS. Phenol red serves as a pH indicator.

The important functions of balanced salt solutions are listed hereunder:

i. Supply essential inorganic ions.

ii. Provide the requisite pH.

iii. Maintain the desired osmolality.

iv. Supply energy from glucose.

In fact, balanced salt solutions form the basis for the preparation of complete media with the requisite additions. Further, BSS is also useful for a short period (up to 4 hours) incubation of cells.

The composition of two most widely used BSS namely Earle's BSS and Hank's BSS is given in Table 34.1.

TABLE 34.1 Composition (g/l) of balanced salt solutions (BSS)

Ingradient	*Earle's BSS*	*Hank's BSS*
NaCl	6.68	8.0
KCl	0.4	0.4
$CaCl_2$ (anhydrous)	0.02	0.14
$MgSO_4.7H_2O$	0.2	0.1
$NaHCO_3$	2.2	0.35
$NaH_3PO_4.H_2O$	0.14	—
$Na_2HPO_4.7H_2O$	—	0.09
KH_2PO_4	—	0.06
D-Glucose	1.0	1.0
Phenol red	0.01	0.01
HEPES, Na salt (buffer)	13.02	2.08

Complete Culture Media:

In the early years, balanced salt solutions were supplemented with various nutrients (amino acids, vitamins, serum etc.) to promote proliferation of cells in culture. Eagle was a pioneer in media formulation. He determined (during 1950-60) the nutrient requirements for mammalian cell cultures. Many developments in media preparation have occurred since then. There are more than a dozen media now available for different types of cultures.

Some of them are stated below:

EMEM—Eagle's minimal essential medium

DMEM—Dulbecco's modification of Eagle's medium

CMEM—Glasgow's modification of Eagle's medium

RPMI 1630 and RPMI 1640—Media from Rosewell Park Memorial Institute.

TABLE 34.2 Composition of three commonly used culture media

Component	*Eagle's MEM*	*RPMI 1640*	*Ham's F 12*
Amino acids			
L-Alanine			8.91
L-Arginine HCl	105	200	211
L-Asparagine H_2O		50	15.0
L-Aspartic acid		20	13.3
L-Cystine	24	50	24.0
L-Glutamic acid		20	14.7
L-Glutamine	292	300	146.2
Glycine		10	7.51
L-Histidine HCl H_2O	31	15	21.0
L-Isoleucine	52	50	3.94
L-Leucine	52	50	13.12
L-Lysine	58	40	36.54
L-Methionine	15	15	4.48
L-Phenylalanine	32	15	4.96
L-Proline		20	34.5
L-Serine		30	10.51
L-Threonine	48	20	11.91
L-Tryptophan	10	5	2.042
L-Tyrosine	36	20	5.43
L-Valine	46	20	11.7
Glutathione (red)		1	
L-Hydroxyproline		20	
Vitamins			
D-Biotin		0.2	0.007
Ca D-pantothenate	1	0.25	0.26
Choline chloride	1	3.0	13.96
Folic acid	1	1.0	1.32
i-Inositol	2		18.02
Nicotinamide	1	35	0.037
p-Aminobenzoic acid		1.0	
Pyridoxine HCl		1	0.062
Pyridoxal HCl	1		
Riboflavin	0.1	0.2	0.038
Thiamine HCl	1	1.0	0.34
Vitamin B_{12}		0.005	1.36

Table 34.2 contd. next column

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Inorganic salts			
$CaCl_2.2H_2O$	200		44.1
$CaNO_3.4H_2O$		100	
$CuSO_4.5H_2O$			0.0025
$FeSO_4.7H_2O$			0.83
KCl	400	400	223
$MgSO_4.7H_2O$	220	100	133
NaCl	6800	6000	7599
$NaHCO_3$	2000	2000	1176
$Na_2HPO_4.7H_2O$		1512	268
$NaH_2PO_4.2H_2O$	150		
Other components			
D-Glucose	1000	2000	1801
Phenol red		5.0	1.2
Sodium pyruvate			110
Lipoic acid			0.21
Linoleic acid			0.084
Hypoxanthine			4.08
Putrescine 2HCl			0.16

Amino acids:

All the essential amino acids (which cannot be synthesized by the cells) have to be added to the medium. In addition, even the non-essential amino acids (that can be synthesized by the cells) are also usually added to avoid any limitation of their cellular synthesis. Among the non-essential amino acids, glutamine and/or glutamate are frequently added in good quantities to the media since these amino acids serve as good sources of energy and carbon.

Vitamins:

The quality and quantity of vitamins depends on the medium. For instance, Eagle's MEM contains only water soluble vitamins (e.g. B-complex, choline, inositol). The other vitamins are obtained from the serum added. The medium M 199 contains all the fat soluble vitamins (A, D, E and K) also. In

general, for the media without serum, more vitamins in higher concentrations are required.

Salts:

The salts present in the various media are basically those found in balanced salt solutions (Eagle's BSS and Hank's BSS). The salts contribute to cations (Na^+, K^+, Mg^{2+}, Ca^{2+} etc.) and anions (CI^-, HCO^-_3, SO^{2-}_4, PO^{3-}_4), and are mainly responsible for the maintenance of osmolality. There are some other important functions of certain ions contributed by the salts.

i. Ca^{2+} ions are required for cell adhesion, in signal transduction, besides their involvement in cell proliferation and differentiation.

ii. Na^+, K^+ and CI^- ions regulate membrane potential.

iii. PO^{3-}_4, SO^{2-}_4 and HCO^-_3 ions are involved in the maintenance of intracellular charge; besides serving as precursors for the production of certain important compounds e.g. PO^{3-}_4 is required for ATP synthesis.

Glucose:

Majority of culture media contain glucose which serves as an important source of energy. Glucose is degraded in glycolysis to form pyruvate/lactate. These compounds on their further metabolism enter citric acid cycle and get oxidized to CO_2. However, experimental evidence indicates that the contribution of glucose for the operation of citric acid cycle is very low in vitro (in culture cells) compared to in vivo situation. Glutamine rather than glucose supplies carbon for the operation of citric acid cycle. And for this reason, the cultured cells require very high content of glutamine.

Hormones and growth factors:

For the media with serum, addition of hormones and growth factors is usually not required. They are frequently added to serum-free media.

Other organic supplements:

Several additional organic compounds are usually added to the media to support cultures. These include certain proteins, peptides, lipids, nucleosides

and citric acid cycle intermediates. For serum-free media, supplementation with these compounds is very useful.

Antibiotics:

In the early years, culture media invariably contained antibiotics. The most commonly used antibiotics were ampicillin, penicillin, gentamycin, erythromycin, kanamycin, neomycin and tetracycline. Antibiotics were added to reduce contamination. However, with improved aseptic conditions in the present day tissue culture laboratories, the addition of antibiotics is not required. In fact, the use of antibiotics is associated with several disadvantages.

i. Possibility of developing antibiotic-resistant cells in culture.

ii. May cause anti-metabolic effects and hamper proliferation.

iii. Possibility of hiding several infections temporarily.

iv. May encourage poor aseptic conditions.

The present recommendation is that for the routine culture of cells, antibiotics should not be added. However, they may be used for the development of primary cultures.

Serum:

Serum is a natural biological fluid, and is rich in various components to support cell proliferation. The major constituents found in different types of sera are listed in Table 34.3. The most commonly used sera are calf serum (CS), fetal bovine serum (FBS), horse serum and human serum. While using human serum, it must be screened for viral diseases (hepatitis B, HIV).

TABLE 34.3 Major constituents of serum

Proteins
Albumin
Globulins
Fetuin
Fibronectin
Transferrin
Protease inhibitors
(α_1-antitrypsin)
Amino acids
Almost all the 20
Lipids
Cholesterol
Phospholipids
Fatty acids
Carbohydrates
Glucose
Hexosamine
Other organic compounds
Lactic acid
Pyruvic acid
Polyamines
Urea
Vitamins
Vitamin A
Folic acid
Growth factors
Epidermal growth factor
Platelet-derived growth factor
Fibroblast growth factor
Hormones
Hydrocortisone
Thyroxine
Triiodothyronine
Insulin
Inorganics
Calcium
Sodium
Potassium
Chlorides
Iron
Phosphates
Zinc
Selenium

Approximately 5-20% (v/v) of serum is mostly used for supplementing several media. Some of the important features of the serum constituents are briefly described.

Proteins:

The in vitro functions of serum protein are not very clear. Some of them are involved in promoting cell attachment and growth e.g. fetuin, fibronectin. Proteins increase the viscosity of the culture medium, besides contributing to buffering action.

Nutrients and metabolites:

Serum contains several amino acids, glucose, phospholipids, fatty acids, nucleosides and metabolic intermediates (pyruvic acid, lactic acid etc.). These constituents do contribute to some extent for the nutritional requirements of cells. This may however, be insignificant in complex media with well supplemented nutrients.

Growth factors:

There are certain growth factors in the serum that stimulate the proliferation of cells in the culture:

i. Platelet-derived growth factor (PDGF).

ii. Fibroblast growth factor (FGF).

iii. Epidermal growth factor (EGF).

iv. Vascular endothelial growth factor (VEGF).

v. Insulin-like growth factors (IGF-1, IGF-2).

In fact, almost all these growth factors are commercially available for use in tissue culture.

Hormones:

Hydrocortisone promotes cell attachment, while insulin facilitates glucose uptake by cells. Growth hormone, in association with somatomedins (IGFs), promotes cell proliferation.

Inhibitors:

Serum may also contain cellular growth inhibiting factors. Majority of them are artefacts e.g. bacterial toxins, antibodies. The natural serum also contains a physiological growth inhibitor namely transforming growth factor β (TGF-β). Most of these growth inhibitory factors may be removed by heat inactivation (at 56°C for 30 minutes).

Selection of Medium and Serum:

As already stated, there are around a dozen media for the cell cultures. The selection of a particular medium is based on the cell line and the purpose of culturing. For instance, for chick embryo fibroblasts and HeLa cells, EMEM is used.

The medium DMEM can be used for the cultivation of neurons. A selected list of cells and cell lines along with the media and sera used is given in Table 34.4. In fact, information on the selection of appropriate medium for a particular cell line is available from literature.

TABLE 34.4 A selected list of the cells or cell lines along with the media and serum used for their culture

Cells or cell line	*Medium*	*Serum*
Chick embryo fibroblasts	EMEM	CS
Chinese hamster ovary (CHO)	EMEM, Ham's F12	CS
HeLa cells	EMEM	CS
Human leukemia	RPMI 1640	FB
Mouse leukemia	Fischer's medium, RPMI 1640	FB, HoS
Neurons	DMEM	FB
Mammary epithelium	RPMI 1640, DMEM	FB
Hematopoietic cells	RPMI 1640, Fischer's medium	FB
Skeletal muscle	DMEM, F 12	FB, HoS
Glial cells	MEM, F 12, DMEM	FB
3T3 cells	MEM, DMEM	CS

The selection of serum is also based on the type of cells being cultured.

The following criteria are taken into consideration while choosing serum:

i. Batch to batch variations.

ii. Quality control.

iii. Efficiency to promote growth and preservation of cells.

iv. Sterility.

v. Heat inactivation.

In recent years, there is a tendency to discontinue the use of serum, and switch over to more clearly defined media.

Supplementation of the Medium with Tissue Extracts:

Besides serum, the culture media can also be supplemented with certain tissue extracts and microbial culture extracts. The examples are—chick embryo extract, proteolytic digests of beef heart, bactopeptone, lactalbumin

hydrolysate, tryptose. The chick embryo extract was found to contain both high molecular weight and low molecular weight compounds that support growth and proliferation of cells.

Animal cell culture

Animal cell culture is one of the major tools now in the field of life science. It is the **in-vitro** technique where the cells are grown in the laboratory conditions by providing a proper nutrient source, growth factors and the environmental factors for the cell growth and division. In the Animal cell culture, the cells are obtained by either enzymatic action like **Trypsinization** or mechanically by **Mincing or Chopping**.

The Animal cell culture, the cells form a monolayer in the solid media and suspension in the liquid media. The method of a cell or tissue culture first involves the production of "**Primary cells**" and followed by subculturing or passaging produces "**Secondary cells**" or "**Cell-lines**".

There are many discoveries which led to the invention of the technique named "Animal cell culture". By the discovery of Animal cell culture, there are some other discoveries also which made the practical study of cell culture possible.

Definition of Animal cell culture

Animal cell culture can define as the type of cell culture where the cell grows and multiplies either in a solid or liquid medium as a "**Cell monolayer**" or "**Cell suspension**" respectively to produce the *Primary cells*. Then followed by subculturing of primary cells, it produces *Secondary cells* to a definite cell number for the normal cells and indefinite for the continuous cells.

Types of Animal cell culture

On the basis of cell growth and division, the cell culture is of two types:

1. Primary cell culture

2. Secondary cell culture

PRIMARY CELL CULTURE

It can define as the culturing of the cells from the tissue of the host animal. The cells can obtain directly by the mechanical method and indirectly by the enzymatic action. Once, the cells are obtained, they have to be cultured on a suitable container which is provided with all the nutrients required for the cell division and growth. The growth of primary either occur as "**Adherent monolayer**" on solid medium or as a "**Suspension**" in a liquid medium.

Adherent cells: These cells adhere to the solid surface and produce a cell population in the monolayer pattern. Adherent cells sometimes refer as "**Confluent cell**", where the cells merge or contract to fill the surface area. The properties of the adherent cell include:

- Adherent cells are "Anchorage-dependent".
- Grow as a "Monolayer".
- Growth occurs on a solid surface or we can say "solid media".
- Adherent cells fill the entire surface area of the container or vessel as a monolayer.
- Adherent cells follow the property of "contract inhibition", where the cell itself ceases the growth of more cells to maintain the synchronized state by the chemical signaling which does not permit the overgrowth once a monolayer is formed.

Examples: Fibroblasts and epithelial cells are examples of Adherent cells.

Suspension cells: These are the cells that do not adhere to the surface of the medium provided. Suspension cells are the type of cells that floats as the suspension in the liquid medium. The properties of the adherent cell include:

- Suspension cells are "Anchorage-independent".
- Floats as a "Suspension of cells" in the liquid culture medium.
- Growth occurs in the liquid nutrient medium.
- Suspension cells grow much faster than the adherent cells.

- There is a short lag phase in the Suspension cells.
- Enzyme action is not required for the dissociation of cells.

SECONDARY CELL CULTURE

It can define as the sub culturing of the primary cells from the primary cells which produce secondary cells or cell lines. The passaging or sub culturing of the primary cell results into a phenotypic and genotypic uniformity of the cell population.

By the sub culturing, the cell-line becomes different from the original cell. On the basis of the life span of the cell, the cell lines categorize into two types:

Finite cell lines: In finite cell lines, there is limited cell division and limited life span. Passaging value is less as after some time the cells lose the ability to grow or proliferate and enters into the phase of senescence or ageing.
Example: Normal cells produce finite cell lines.
Continuous cell lines: In continuous cell lines, the number of cell division and passaging value is indefinite. The passaging value is more for the continuous cells which do not lose the ability to divide i.e. these can grow and divide by an infinite number of times.
Example: Cancerous cells produce Infinite or continuous cell lines.
Process of Animal cell culture

The process of animal cell culture includes the following steps:

1. Tissue explant
2. Cell extraction
3. Culturing in a nutrient medium
4. Sub culturing

Therefore, the process of animal cell culture can be summarized in the following way:

Tissue explants: The removal of tissue from the organ refers to as "Tissue explant".

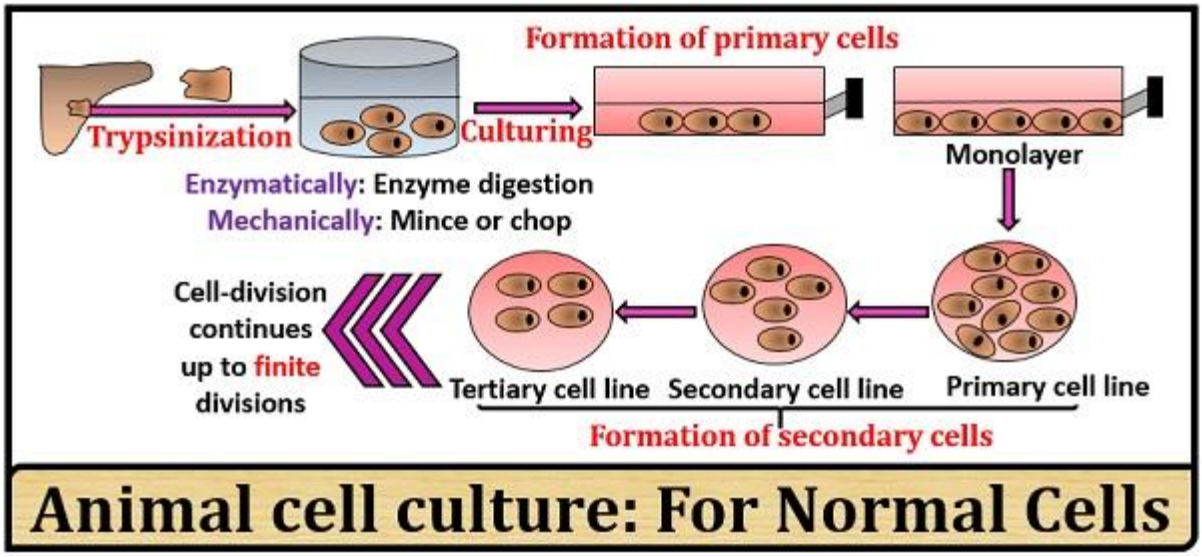
Cell extraction: The cell can extract from the tissue either mechanically or enzymatically. The extraction is mostly carried out by the enzyme action or by the process of "Trypsinization".

Culturing in a nutrient medium: After that, the cell is cultured either on solid nutrient medium or liquid nutrient medium.

In a solid nutrient medium, the primary cells form a monolayer whereas in liquid medium primary cells appears as a cell suspension.

Subculturing: It also refers to as "**Passaging**" of the cell. After the formation of primary cells, the subculturing is carried out that is important to continuously study or to grow the cells. This is the most important step in cell culture, which helps us to understand the cell type.

***Normal cell*:** It has low passaging value because these lose their ability to divide after some time due to cell ageing. Therefore, the cell divides to produce definite cell lines.



***Continuous cell*: It** has high passaging value because as from the name it is clear that these kind of cells are having an ability to continuously divide. Therefore, the cell divides to produce indefinite cell lines.

Transformation of Cells:

Transformation broadly refers to the change in phenotype of a cell due to a new genetic material. As regards the cultured cells, transformation involves spontaneous or induced permanent phenotypic alterations as a result of heritable changes in DNA, and consequently gene expression.

Transformation of cells may occur due to any one of the following causes that ultimately result in a changed genetic material:

i. Spontaneous.

ii. Infection with transforming virus.

iii. From gene transfection.

iv. Exposure to chemical carcinogens.

v. Exposure to ionizing radiations.

Characteristics of Transformed Cells:

The general characters of transformed cells are given in Table 39.1. They are grouped as genetic, structural, growth and neoplastic, and listed.

TABLE 39.1 General characteristics of transformed cells

Genetic characters
Aneuploid
Heteroploid
High spontaneous mutation rate
Overexpressed oncogenes
Mutated or deleted suppressor genes
Structural characters
Altered cytoskeleton
Changed extracellular matrix
Modified expression of cell adhesion molecules
Disrupted cell polarity
Growth characters
Immortalized cells
Loss of contact inhibition
Anchorage independent
Density limitation of growth reduced
Growth factor independent
Low serum requirement
Shorter population doubling time
Neoplastic characters
Tumorigenic
Invasive
Increased protease secretion

Transformation is associated with genetic instability, immortalization, aberrant growth control and malignancy. These aspects are briefly described.

Genetic Instability:

In general, the cell lines in culture are prone to genetic instability. A majority of normal finite cell lines are usually genetically stable while cell lines from other species (e.g. mouse) are genetically unstable, and can get easily transformed. The continuous cell lines derived from tumors of all species are unstable.

The normally occurring genetic variations in the cultured cells are due to the following causes:

1. High rate of spontaneous mutations in the in vitro conditions, possibly due to high rate of cell proliferation.

2. The continued presence of mutant cells in the culture, as they are not normally eliminated.

cell lines

A cell line is a permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space. **Lines** differ from **cell** strains in that they become immortalized.

The following two approaches are in use to understand malignant-associated properties of cultured cells:

1. The cells can be cultured from malignant tumors and characterized.

2. Viral genes or chemical carcinogens can be used to transform the untransformed cell.

TYPES OF CELL LINES

Finite Cell Lines:

The cells in culture divide only a limited number of times, before their growth rate declines and they eventually die. The cell lines with limited culture life spans are referred to as finite cell lines. The cells normally divide 20 to 100 times (i.e. is 20-100 population doublings) before extinction. The actual number of doublings depends on the species, cell lineage differences, culture conditions etc. The human cells generally divide 50-100 times, while murine cells divide 30-50 times before dying.

Continuous Cell Lines :

A few cells in culture may acquire a different morphology and get altered. Such cells are capable of growing faster resulting in an independent culture. The progeny derived from these altered cells has unlimited life (unlike the cell strains from which they originated). They are designated as continuous cell lines.

The continuous cell lines are transformed, immortal and tumorigenic. The transformed cells for continuous cell lines may be obtained from normal primary cell cultures (or cells strains) by treating them with chemical carcinogens or by infecting with oncogenic viruses. In the Table. 36.1, the different properties of finite cell lines and continuous cell lines are compared.

Table 36.1 Comparison of properties of finite and continuous cell lines

Property	*Finite cell line*	*Continuous cell line*
Growth rate	Slow	Fast
Mode of growth	Monolayer	Suspension or monolayer
Yield	Low	High
Transformation	Normal	Immortal, tumorigenic
Ploidy	Euploid (multiple of haploid chromosomes)	Aneuploid (not an exact multiple of haploid chromosomes)
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Cloning efficiency	Low	High
Serum requirement	High	Low
Markers	Tissue specific	Chromosomal, antigenic or enzymatic

The most commonly used terms while dealing with cell lines are explained below.

Split ratio:

The divisor of the dilution ratio of a cell culture at subculture. For instance, when each subculture divided the culture to half, the split ratio is 1: 2.

Passage number:

It is the number of times that the culture has been sub-cultured.

Generation number:

It refers to the number of doublings that a cell population has undergone. It must be noted that the passage number and generation number are not the same, and they are totally different.

Nomenclature of Cell Lines:

It is a common practice to give codes or designations to cell lines for their identification. For instance, the code NHB 2-1 represents the cell line from normal human brain, followed by cell strain (or cell line number) 2 and clone number 1. The usual practice in a culture laboratory is to maintain a log book or computer database file for each of the cell lines.

While naming the cell lines, it is absolutely necessary to ensure that each cell line designation is unique so that there occurs no confusion when reports are given in literature. Further, at the time of publication, the-cell line should be prefixed with a code designating the laboratory from which it was obtained e.g. NCI for National Cancer Institute, Wl for Wistar Institute.

Commonly used cell lines:

There are thousands of cell lines developed from different laboratories world over. A selected list of some commonly used cell lines along with their origin, morphology and other characters are given in Table. 36.2.

Table 36.2 A selected list of commonly used cell lines

Cell line	*Species of origin*	*Tissue of origin*	*Morphology*	*Ploidy*	*Characteristics*
IMR-90	Human	Lung	Fibroblast	Diploid	Susceptible to human viral infections.
3T3-A31	Mouse	Connective tissue	Fibroblast	Aneuploid	Contact inhibited, readily transformed
BHK21-C13	Hamster (Syrian)	Kidney	Fibroblast	Aneuploid	Readily transformable
CHO-k1	Chinese hamster	Ovary	Fibroblast	Diploid	Simple karyotype
NRK49F	Rat	Kidney	Fibroblast	Aneuploid	Induction of suspension growth by TGF-α, β.
BRL 3A	Rat	Liver	Epithelial	Diploid	Produces IGF-2
Vero	Monkey	Kidney	Fibroblast	Aneuploid	Viral substrate and assay
HeLa-S_3	Human	Cervical carcinoma	Epithelial	Aneuploid	Rapid growth, high plating efficiency.
Sk/HEP-I	Human	Hepatoma	Endothelial	Aneuploid	Factor VIII
Caco-2	Human	Colo-rectal carcinoma	Epithelial	Aneuploid with polarised support.	Forms tight monolayer
MCF-7	Human	Breast tumor (effusion)	Epithelial	Aneuploid	Estrogen receptor positive.
Friend	Mouse	Spleen	Suspension	Aneuploid	Hemoglobin, growth hormone.

Selection of Cell Lines:

Several factors need to be considered while selecting a cell line.

1. Species:

In general, non-human cell lines have less risk of biohazards, hence preferred. However, species differences need to be taken into account while extrapolating the data to humans.

2. Finite or continuous cell lines:

Cultures with continuous cell lines are preferred as they grow faster, easy to clone and maintain, and produce higher yield. But it is doubtful whether the continuous cell lines express the right and appropriate functions of the cells. Therefore, some workers suggest the use of finite cell lines, although it is difficult.

3. Normal or transformed cells:

The transformed cells are preferred as they are immortalized and grow rapidly.

4. Availability:

The ready availability of cell lines is also important. Sometimes, it may be necessary to develop a particular cell line in a laboratory.

5. Growth characteristics:

The following growth parameters need to be considered:

i. Population doubling time

ii. Ability to grow in suspension

iii. Saturation density (yield per flask)

iv. Cloning efficiency.

6. Stability:

The stability of cell line with particular reference to cloning, generation of adequate stock and storage are important.

INSECT CELL LINES

Insect's cell lines are commonly used in place of prokaryotic ones because post-translational modifications of proteins are possible in insect cells whereas this mechanism is not present in prokaryotic systems. The Sf9 cell line is one of the most commonly used lines in insect cell culture.

Insect cells can efficiently express recombinant proteins and are mostly used for the development of virus-like particles and vaccines. It has been proven that insect cells are excellent platforms for the production of recombinant antibodies. Creative Biolabs can provide different insect systems to allow constitutive or inducible production of recombinant proteins.

For recent years, insect cells have been extensively used for the production of different classes of biologically active recombinant proteins. There are mainly three insect expression systems: baculovirus-insect cell system (BEVS), InsectSelect (IS) system and *Drosophila* expression system (DES).

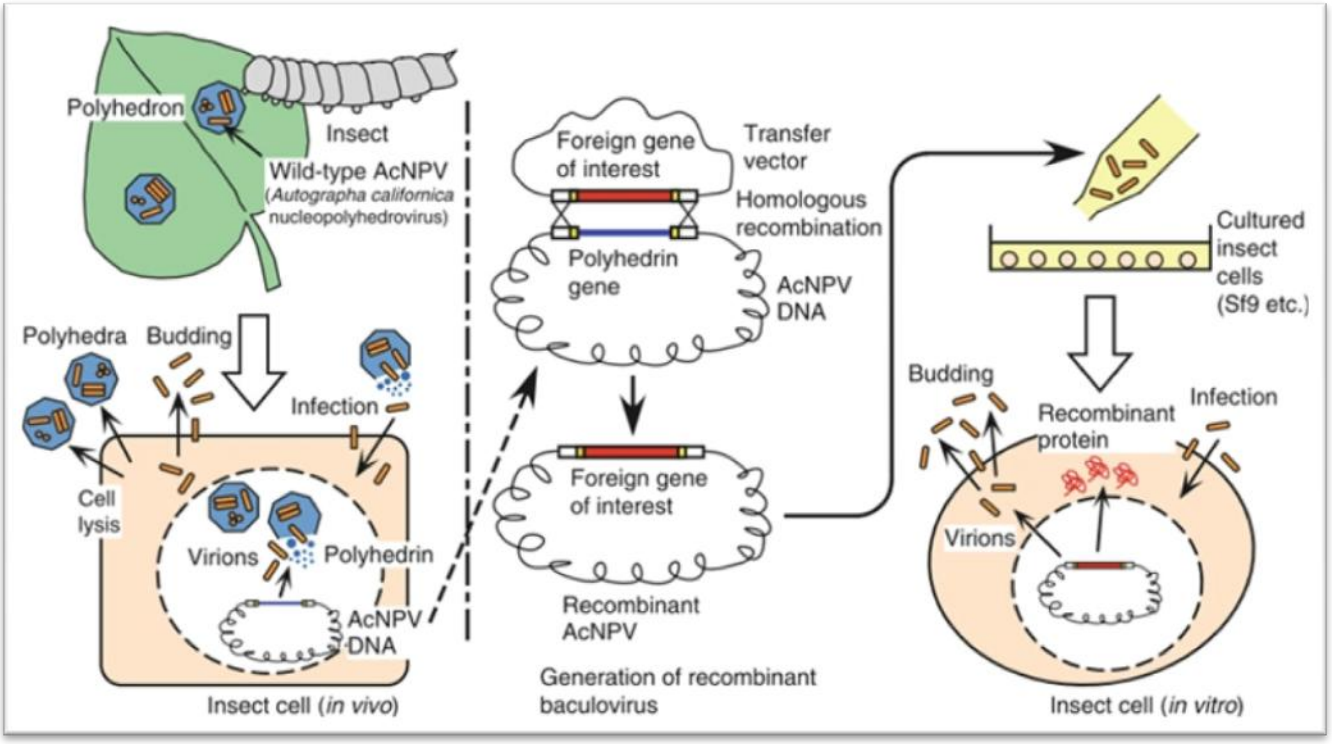


Figure: Recombinant protein production in the baculovirus-insect cell system (*Antibody Expression and Production, 2010*)

The baculovirus-insect cell system is the most widely used insect cell-based expression system especially for transient expression. Baculovirus are rod-shaped viruses, which have a large, circular double-stranded DNA genome. These viruses exclusively infect arthropods, mainly insects. In baculovirus-insect cell system, the commonly used baculoviruses are in the genus *Nucleopolyhedrovirus* including AcNPV (*Autographa alifornica* nucleopolyhedrovirus) and BmNPV (*Bombyx mori* nucleopolyhedrovirus). During the infection of *Nucleopolyhedrovirus,* there are immense quantities of two viral proteins synthesized by infected cells, polyhedron and p10

Polyhedrin molecules assemble into polyhedral. Polyhedron and p10 are not essential for replication of the virus. Moreover, the promoter of these two genes is remarkably strong. Therefore, the polyhedrin or/and p10 gene/genes can be replaced with the gene of interest to construct a recombinant nucleo polyhedrovirus to obtain large quantities of products. In order to improve baculovirus expression vectors, two modifications have been established: transfer plasmid modification and parental baculovirus genome modifications. Various forms of recombinant proteins have been expressed at high yields using baculovirus-insect cell system, including whole antibodies, Fab fragments, scFv fragments and related proteins.

In insect systems, insect larvae, pupae and cell lines can all work as hosts. Sf21, Sf9 and BTI-TN-5B1-4 (High Five) are most commonly used with AcNPV. The three cell lines are derived from lepidopteran insects. Sf21 and Sf9 are derived from the pupal ovarian tissue of *Spodoptera frugiperda,* a fall army worm. BTI-TN-5B1-4 (High Five) is from the ovarian cells of *Trichoplusia ni*, cabbage looper. While transformed stable insect cell lines are often from dipteraninsects including fruit flies and mosquitoes. Among which, the cell line *Drosophila melanogaster* Schneider 2 (S2) has been most commonly used. In the system, an antibiotic resistance gene using as a selectable marker is co-transfected with the heterologous gene of interest. *Drosophila* metallothionein is often used as the promoter for inducible expression.

Drosophila actin 5C promoter has been demonstrated for constitutive secretory expression in S2. Furthermore, the mentioned lepidopteran insect cells, Sf9 and High Five can also been used to establish stable cell lines, among which the choice of a promoter is crucial. The combination of AcNPV hr5 enhancer and AcNPV-immediated early promoter IE1, BmNPV IE-1 transactivator, BmNPV HR3 enhancer, and B. mori actin promoter with either a blasticidin ora neomycin resistance gene have been reported in stably transformed High Five. And OpNPV immediate early 2 promoter OpIE2 is used in Sf9.

Based on the recombinant proteins of interest and the special requirements of client, Creative Biolabs provides insect systems including transient and stable expression systems to get high-quality products at high yields.

STEM CELL CULTURE

Stem cells are the roots from which different cell lineages arise, and they exist in multiple forms. Inducible pluripotent stem cells (iPSCs) are ESC-like cells that are created from somatic (differentiated) cells using specific transcription factors or chemicals **There are three types of stem cells**: adult stem cells, embryonic (or pluripotent) stem cells, and induced pluripotent stem cells (iPSCs).

CYTOTOXICITY AND CELL VIABILITY ASSAYS

Cytotoxicity is one of the most important indicators for biological evaluation in vitro studies. In vitro, chemicals such as drugs and pesticides have different cytotoxicity mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. In order to determine the cell death caused by these damages, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays.

CYTOTOXICITY AND CELL VIABILITY ASSAYS

Cytotoxicity and cell viability assays are based on various cell functions. A broad spectrum of cytotoxicity assays is currently used in the fields of toxicology and pharmacology.

There are different classifications for these assays:

(i) dye exclusion assays

(ii) colorimetric assays

(iii) fluorometric assays

(iv) luminometric assays.

Choosing the appropriate method among these assays is important for obtaining accurate and reliable results. When selecting the cytotoxicity and cell viability assays to be used in the study, different parameters have to be considered such as the availability in the laboratory where the study is to be performed, test compounds, detection mechanism, specificity, and sensitivity. In this chapter, information will be given about in vitro cytotoxicity and viability assays, these assays will be classified and their advantages and disadvantages will be emphasized. The aim of this chapter is to guide the researcher interested in this subject to select the appropriate assay for their study.

Viability levels and/or proliferation rates of cells are good indicators of cell health. Physical and chemical agents can affect cell health and metabolism. These agents may cause toxicity on cells via different mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors, inhibition of polydeoxynucleotide elongation, and enzymatic reactions. In order to determine the cell death caused by these mechanisms, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays.

In vitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Application of these assays has been of increasing interest over recent years.

Currently, these assays are also used in oncological researches to evaluate both compound toxicity and tumor cell growth inhibition during drug development. Because, they are rapid, inexpensive and do not require the use of animals. Furthermore, they are useful for testing large number of samples. Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity.

In vitro cytotoxicity and/or cell viability assays have some advantages, such as speed, reduced cost and potential for automation, and tests using human cells may be more relevant than some in vivo animal tests. However, they have some disadvantages because they are not technically advanced enough yet, to replace animal tests.

It is important to know how many viable cells are remaining and/or how many cells are dead at the end of the experiment. A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. The choice of assay method is crucial in the assessment of the interaction type.

2. Classification of cytotoxicity and cell viability assays

Although there are different classifications for cytotoxicity and cell viability assays, in this chapter, these assays are classified according to measurement types of end points (color changes, fluorescence, luminescent etc.).

Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.

Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.

Fluorometric assays: alamar Blue assay and CFDA-AM assay.

Luminometric assays: ATP assay and real-time viability assay.

Dye exclusion assays

The proportion of viable cells in a cell population can be estimated in various methods. The simplest and widely used one of the methods is dye exclusion method. In dye exclusion method, viable cells exclude dyes, but dead cells not exclude them. Although the staining procedure is quite simple, experimental procedure of large number of samples is difficult and time consuming. Determination of membrane integrity is possible via dye exclusion method. A

variety of such dyes have been employed, including eosin, Congo red, erythrosine B, and trypan blue of the dyes listed, trypan blue has been used the most extensively.

If dye exclusion assays are used, following factors must be considered

(i) Lethally damaged cells by cytotoxic agents may require several days to lose their membrane integrity

(ii) The surviving cells may continue to proliferate during this time, and

(iii) Some lethally damaged cells are not appear to be stained with dye at the end of the culture period, because they may undergo an early disintegration. Factors (ii) and (iii) may cause an underestimate of cell death when the results of the assay are based on percent viability expression.

Dye exclusion assays have unique advantages for chemo sensitivity testing. They are comparatively simple, require small numbers of cells, are rapid, and are capable of detecting cell kill in nondividing cell populations. Further investigations into the possible role of these assays in chemosensitivity testing are warranted.

However, none of these dyes is recommended for use on monolayer cell cultures but rather they are intended for cells in suspension; thus monolayer cells must first trypsinized.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most commonly used colori meteric assay to assess cytotoxicity or cell viability. This assay determines principally cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial enzymes such as succinate dehydrogenase. In this assay, MTT is reduced to a purple formazan by NADH. This product can be quantified by light absorbance at a specific wavelength.

Advantages: This method is far superior to the previously mentioned dye exclusion methods because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests.

Disadvantages: MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, prior to measuring the absorbance, an organic solvent such as dimethyl sulfoxide (DMSO) or isopropanol is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error.

Additional control experiments should be conducted to reduce false-positive or false-negative results that caused by background interference due to inclusion of particles. This interference could lead to an overestimation of the cell viability. This can often be controlled by subtraction of the background absorbance of the cells in the presence of the particles, but without the assay reagents.

BIOLOGY OF CULTURED CELLS

The culture environment:

The validity of the cultured cell as a model of physiological function in vivo has frequently been criticized. Often, the cell does not express the correct in vivo phenotype because the cell's microenvironment has changed. Cell–cell and cell–matrix interactions are reduced because the cells lack the heterogeneity and three-dimensional architecture found in vivo, and many hormonal and nutritional stimuli are absent. This creates an environment that favors the spreading, migration, and proliferation of unspecialized progenitor cells, rather than the expression of differentiated functions. The influence of the environment on the culture is expressed via four routes: (1) the nature of the

substrate on or in which the cells grow—solid, as on plastic or other rigid matrix, semisolid, as in a gel such as collagen or agar, or liquid, as in a suspension culture; (2) the degree of contact with other cells; (3) the physicochemical and physiological constitution of the medium; (4) the constitution of the gas phase; and (5) the incubation temperature. The provision of the appropriate environment, including substrate adhesion, nutrient and hormone or growth factor concentration, and cell interaction, is fundamental to the expression of specialized functions

Characteristics of Cultured Cells:

Some of the important distinguishing properties of cultured cells are given below:

1. Cells which do not normally proliferate in vivo can be grown and proliferated in cultures.

2. Cell to cell interactions in the cultured cells are very much low.

3. The three dimensional architecture of the in vivo cells is not found in cultured cells.

4. The hormonal and nutritional influence on the cultured cells differs from that on the in vivo cells.

5. Cultured cells cannot perform differentiated and specialized functions.

6. The environment of the cultured cells favours proliferation and spreading of unspecialized cells.

Environmental influence on cultured cells:

The environmental factors strongly influence the cells in culture. The major routes through which environmental influence occurs are listed:

i. The nature of the substrate or phase in which cells grow. For monolayer cultures, the substrate is a solid (e.g. plastic) while for suspension cultures, it is a liquid.

ii. The composition of the medium used for culture nutrients and physicochemical properties.

iii. Addition of hormones and growth factors.

iv. The composition of the gas phase.

v. The temperature of culture incubation.

The biological and other aspects of cultured cells with special reference to the following parameters are briefly described:

1. Cell adhesion.

2. Cell proliferation.

3. Cell differentiation.

4. Metabolism of cultured cells.

5. Initiation of cell culture.

6. Evolution and development of cell lines.

Cell Adhesion:

Most of the cells obtained from solid tissues grow as adherent monolayers in cultures. The cells, derived from tissue aggregation or subculture, attach to the substrate and then start proliferating. In the early days of culture techniques, slightly negatively charged glasses were used as substrates. In recent years, plastics such as polystyrene, after treatment with electric ion discharge, are in use.

The cell adhesion occurs through cell surface receptors for the molecules in the extracellular matrix. It appears that the cells secrete matrix proteins which spread on the substrate. Then the cells bind to matrix through receptors. It is a common observation that the substrates (glass or plastic) with previous cell culture are conditioned to provide better surface area for adhesion.

Cell adhesion molecules:

Three groups of proteins collectively referred to as cell adhesion molecules (CAMs) are involved in the cell-cell adhesion and cell-substrate adhesion.

Cell-cell adhesion molecules:

These proteins are primarily involved in cell-to-cell interaction between the homologous cells. CAMs are of two types — calcium-dependent ones (cadherin's) and calcium-independent CAMs.

Integrin's:

These molecules mediate the cell substrate interactions. Integrin's possess receptors for matrix molecules such as fibronectin and collagen.

Proteoglycans:

These are low affinity trans membrane receptors. Proteoglycans can bind to matrix collagen and growth factors. Cell adhesion molecules are attached to the cytoskeletons of the cultured cells.

Measurement of Growth Parameters of Cultured Cells:

Information on the growth state of a given culture is required to:

a. Design culture experiments.

b. Routine maintenance of culture.

c. Measurement of cell proliferation.

d. Know the time for subculture.

e. Determine the culture response to a particular stimulus or toxin.

Some of the commonly used terms in relation to the measurement of growth of cultured cells are explained.

Population doubling time (PDT):

The time interval for the cell population to double at the middle of the logarithmic (log) phase.

Cell cycle time or generation time:

The interval from one point in the cell division to the same point in the cycle, one division later. Thus cell cycle time is measured form one point in the cell cycle until the same point is reached again.

Confluence:

It denotes the culture stage wherein all the available substrate (growth area) is utilized, and the cells are in close contact with each other.

Contact inhibition:

Inhibition of cell motility and plasma membrane ruffling when the cells are in complete contact with other adjacent cells. This mostly occurs at confluence state, and results in the ceasation of the cell proliferation.

Cell density:

The number of cells per ml of the medium.

Saturation density:

The density of the cells (cells/ml^2, surface area) in the plateau phase.

Growth Cycle of Cultured Cells:

The growth cycle of cultured cells is conventionally represented by three phases — the lag phase, the log (exponential) phase and the plateau phase (Fig. 35.5). The properties of the cultured cells vary in the phases.

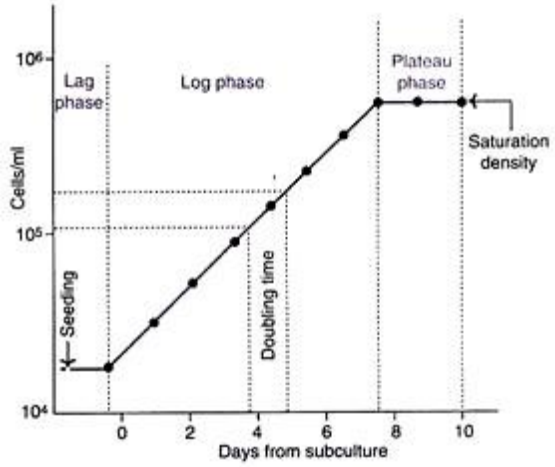


*Fig. 35.5 : Growth curve of cultured cells (**Note** : The cell concentration is expressed in semilog plot).*

The lag phase:

The lag phase represents a period of adaptation during which the cell forms the cell surface and extracellular matrix (lost during trypsinization), attaches to the substrate and spreads out. There is an increased synthesis of certain enzymes (e.g. DNA polymerase) and structural proteins, preparing the cells for proliferation.

The production of specialized products disappears which may not reappear until the cell proliferation ceases. The lag phase represents preparative stage of the cells for proliferation following subculture and reseeding.

The log phase:

The log phase is characterized by an exponential growth of cells, following the lag phase.

The duration of log phase depends on the cells with reference to:

a. Seeding density.

b. Growth rate.

c. Density after proliferation.

During the log phase, the cultured cells are in the most uniform and reproducible state with high viability. This is an ideal time for sampling. The log phase terminates after confluence is reached with an addition of one or two population doublings.

The plateau phase:

As the cells reach confluence, the growth rate is much reduced, and the proliferation of cultured cells almost stops.

This stage represents plateau or stationary phase, and is characterized by:

a. Low motility of cells.

b. Reduced ruffling of plasma membrane.

c. Cells occupying minimum surface area.

d. Contact inhibition.

e. Saturation density.

f. Depletion of nutrients and growth factors.

g. Reduced synthesis of structural proteins.

h. Increased formation of specialized products.

The majority of normal cultured cells that form monolayers stop growing as they reach confluence. Some of the cells however, with replenishment of

medium continue to grow (at a reduced rate) after confluence, forming multilayers of cells. The transformed cultured cells usually reach a higher cell density compared to the normal cells in the plateau phase (Fig. 35.6).

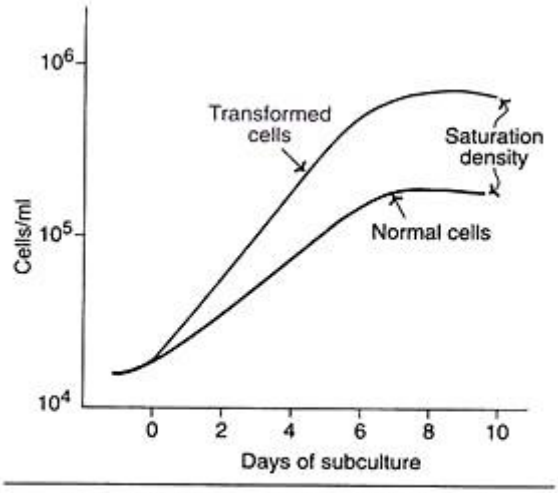


*Fig. 35.6 : Growth curves of transformed and normal cells (**Note** : The cell concentration is expressed in semilog plot).*

Plating Efficiency of Cultured Cells:

Plating efficiency, representing colony formation at low cell density, is a measure used for analyzing cell proliferation and survival.

When the cells, at low densities, are cultured in the form of single cell suspensions, they grow as discrete colonies. Plating efficiency is calculated as follows.

Plating efficiency = No. of colonies formed/No. of cells seeded × 100

The term cloning efficiency is used (instead of plating efficiency) when each colony grows from a single cell.

Seeding efficiency representing the survival of cells at higher densities is calculated as follows.

Seeding efficiency = No. of cells recovered/No. of cells seeded × 100

Cell Synchronization:

Synchronization literally means to make two or more things happen exactly simultaneously. For instance, two or more watches can be synchronized to show exactly the same time. The cells at different stages of the cell cycle in a culture can be synchronized so that the cells will be at the same phase. Cell synchrony is required to study the progression of cells through cell cycle. Several laboratory techniques have been developed to achieve cell synchronization.

They are broadly categorized into two groups:

1. Physical fractionation for cell separation.

2. Chemical blockade for cell separation.

Cell Separation by Physical Means:

Physical fractionation or cell separation techniques, based on the following characteristics are in use:

a. Cell density.

b. Cell size.

c. Affinity of antibodies on cell surface epitopes.

d. Light scatter or fluorescent emission by labeled cells.

The two commonly used techniques namely centrifugal elutriation and fluorescence-activated cell separation are briefly described hereunder.

Centrifugal elutriation:

The physical characteristics—cell size and sedimentation velocity are operative in the technique of centrifugal elutriation. Centrifugal elutriator (from

Beckman) is an advanced device for increasing the sedimentation rate so that the yield and resolution of cells is better. The cell separation is carried out in a specially designed centrifuge and rotor (fig. 35.7). The cells in the medium are pumped into the separating chamber while the rotor is turning.

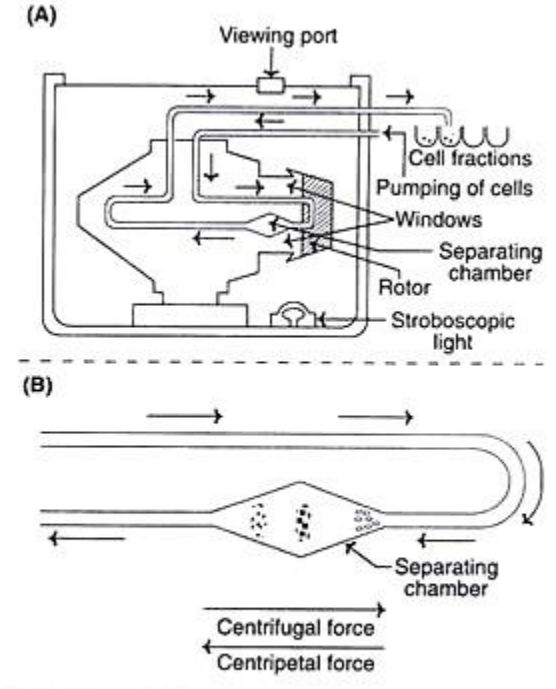


Fig. 35.7 : (A) Diagrammatic view of a centrifugal elutriator, (B) Separation chamber of elutriator.

Due to centrifugal force, the cell will be pushed to the edges. As the medium is then pumped through the chamber in such a way that the centripetal flow is equal to the sedimentation rate of cells. Due to differences in the cells (size, density, cell surface configuration), the cells tend to sediment at different rates, and reach equilibrium at different positions in the chamber.

The entire operation in the elutriator can be viewed through the port, as the chamber is illuminated by stroboscopic light. At the equilibrium the flow rate can be increased and the cells can be pumped out, and separated in collecting vessels in different fractions. It is possible to carry out separation of cells in a complete medium, so that the cells can be directly cultured after separation.

Fluorescence-activated cell sorting:

Fluorescence-activated cell sorting is a technique for sorting out the cells based on the differences that can be detected by light scatter (e.g. cell size) or fluorescence emission (by pretreated DNA, RNA, proteins, antigens). The procedure involves passing of a single stream of cells through a laser beam so that the scattered light from the cells can be detected and recorded. When the cells are pretreated with a fluorescent stain (e.g. chromomycin A for DNA), the fluorescent emission excited by the laser can be detected.

There are two instruments in use based on the principle of fluorescent-activated cell sorting:

1. Flow cytometer:

This instrument is capable of sorting out cells (from a population) in different phases of the cell cycle based on the measurements of a combination of cell size and DNA fluorescence.

2. Fluorescent-activated cell sorter (FACS):

In this instrument, the emission signals from the cells are measured, and the cells sorted out into collection tubes.

Some Highlights of Cell Synchronization:

a. Cell separation by physical methods is more effective than chemical procedures.

b. Chemical blockade is often toxic to the cells.

c. Transformed cells cannot be synchronized by nutritional deprivation.

d. A high degree of cell synchrony (>80%) can be obtained in the first cycle, and in the second cycle it would be <60%. The cell distribution may occur randomly in the third cycle.

Cellular Senescence and Apoptosis:

As the cells grow in culture, they become old due to aging, and they cannot proliferate any more. The end of the proliferative life span of cells is referred to as senescence.

Cellular Senescence:

The growth of the cells is usually measured as population doublings (PDs). The PDs refer to the number of times the cell population doubles in number during the period of culture and is calculated by the following formula.

Log_{10} (No. of cells harvested) – log_{10} (No. of cells seeded)/ $log10^2$

The phenomenon of senescence has been mostly studied with human fibroblast cultures. After 30-60 populations doublings, the culture is mainly composed of senescent fibroblasts. These senescent fibroblast are unable to divide in response to mitotic stimuli. It must be noted that the cells do not appear suddenly, but they gradually accumulate and increase in number during the life span of the culture.

The different parameters used for the measurement of cell growth in cultures are listed below:

a. Direct measure of cell number.

b. Determination of DNA/RNA content.

c. Estimation of protein/ATP concentration.

Measurement of Senescence:

The direct measurement of senescent cells is rather difficult.

Some of the indirect measures are:

a. Loss of metabolic activity

b. Lack of labeled precursor (^{3}H-thymidine) incorporation into DNA.

c. Certain histochemical techniques.

Senescence-associated β-galactosidase activity assay

There occurs an overexpression of the lysosomal enzyme β-galactosidase at senescence. This enzyme elevation is also associated with an increase in the cell size as the cell enters a permanent non-dividing state. The number of senescent cells in a culture can be measured by senescence-associated β-galactosidase (SA-β) assay.

The assay consists of the following stages:

1. Wash the cells and fix them using a fixative (e.g. para formaldehyde), and wash again.

2. Add the staining solution (X-gal powder in dimethylformamide dissolved in buffer) to the fixed cells and incubate.

3. The senescent cells display a dense blue colour which can be counted.

Apoptosis:

The process of programmed cell death (PCD) is referred to as apoptosis. The cell death may be initiated by a specific stimulus or as a result of several signals received from the external environment. Apoptosis occurs as a result of inherent cellular mechanisms, which finally lead to self-destruction. The cell activates a series of molecular events that cause an orderly degradation of the cellular constituents with minimal impact on the neighboring tissues.

Reasons for in situ apoptosis:

1. for proper development:

The formation of fingers and toes of the fetus requires the removal of the tissues between them. This is usually carried out by apoptosis.

2. Destruction of cells that pose threat to the integrity of the organism:

Programmed cell death is needed to destroy and remove the cells that may otherwise damage the organisms.

Mechanism of apoptosis:

The programmed cell death may occur due to three different mechanisms:

1. Apoptosis due to internal signals.

2. Apoptosis triggered by external signals e.g. tumor necrosis factor-α (TNF-α), lymphotoxin.

3. Apoptosis triggered by reactive oxygen species.

Measurement of Apoptosis:

A simple and easy way of detecting dead or dying cells is the direct microscopic observation. The dying cells are rounded with dense bodies which can be identified under phase contrast microscope. The cells that have undergone apoptosis contain fragmented chromatin which can be detected by conventional staining techniques. In recent years, more sensitive and reliable techniques have been developed for measuring apoptosis.

Some of them are briefly described:

Determination ADP/ATP ratio:

Both the growth and apoptosis of cells require ATP. But when there is growth arrest, an elevation of ADP occurs. Thus measuring ADP/ATP ratio will throw light on the dead cells. In fact, some assay systems for measuring ADP/ATP ratios are commercially available.

TUNEL assay:

A significant biochemical event for the apoptosis is the activation of endogenous nuclease activity. This enzyme cleaves DNA into fragments with free 3-hydroxyl groups. The newly formed small DNA fragments can be extended by employing the enzyme DNA polymerase. If labeled nucleotides are used for DNA fragment extension, they can be detected.

TUNEL is an abbreviation for TdT-mediated dUTP nick end-labeling assay. TUNEL is very fast and effective for the determination of DNA fragments formed by endogenous nuclease activity. The apoptotic nuclei can be identified by a fluorescent technique using fluorescein isothiocyanate (FITC) and 4, 6-diaminophenylindole.

DNA laddering test:

During the course of apoptosis, the genomic DNA is cleaved to mono — and oligonucleosomal DNA fragments. These fragments can be separated by agarose electrophoresis, and detected. The nucleosomal fragments of apoptotic cells give a characteristic ladder pattern on electrophoresis.

Limitations of the test:

DNA laddering test is not very specific since several cells that have undergone apoptosis may not show DNA laddering. Further, some cells not subjected to apoptosis may also show DNA ladders, for these reasons, DNA laddering test is coupled with some other test for measurement of apoptosis.

er test applications to humans as a cure, a way to transform human stem cells into heart tissue would have to be found

In 2015, Harald Ott was able to grow a rat. Forelimb He now works at Ott Lab which focuses on the creation of bioartificial hearts, lungs, tracheas and kidneys.

In vitro culture

Embryonic organ culture is an easier alternative to normal organ culture derived from adult animals. The following are four techniques employed for embryonic organ culture.

Plasma clot method

The following are general steps in organ culture on plasma clots.

1. Prepare a plasma clot by mixing 15 drops of plasma with five drops of embryo extract in a watch glass.
2. Place a watch glass on a pad of cotton wool in a petri dish; cotton wool is kept moist to prevent excessive evaporation from the dish.
3. Place a small, carefully dissected piece of tissue on top of the plasma clots in watch glass.

The technique has now been modified, and a raft of lens paper or rayon net is used on which the tissue is placed. Transfer of the tissue can then be achieved by raft easily. Excessive fluid is removed and the net with the tissue placed again on the fresh pool of medium.

Agar gel method

Media solidified with agar are also used for organ culture and these media consist of 7 parts 1% agar in BSS, 3 parts chick embryo extract and 3 parts of horse serum. Defined media with or without serum are also used with agar. The medium with agar provides the mechanical support for organ culture. It does not liquefy. Embryonic organs generally grow well on agar, but adult organ culture will not survive on this medium.

The culture of adult organs or parts from adult animals is more difficult due to their greater requirement of oxygen. A variety of adult organs (e.g. the liver) have been cultured using special media with special apparatus (Towell's II

culture chamber). Since serum was found to be toxic, serum-free media were used, and the special apparatus permitted the use of 95% oxygen.

Raft Methods

In this approach the explant is placed onto a raft of lens paper or rayon acetate, which is floated on serum in a watch glass. Rayon acetate rafts are made to float on the serum by treating their 4 corners with silicone.

Similarly, floatability of lens paper is enhanced by treating it with silicone. On each raft, 4 or more explants are usually placed.

In a combination of raft and clot techniques, the explants are first placed on a suitable raft, which is then kept on a plasma clot. This modification makes media changes easy, and prevents the sinking of explants into liquefied plasma.

Grid Method

Initially devised by Trowell in 1954, the grid method utilizes 25 mm x 25 mm pieces of a suitable wire mesh or perforated stainless steel sheet whose edges are bent to form 4 legs of about 4 mm height.

Skeletal tissues are generally placed directly on the grid but softer tissues like glands or skin are first placed on rafts, which are then kept on the grids.

The grids themselves are placed in a culture chamber filled with fluid medium up to the grid; the chamber is supplied with a mixture of O_2 and CO_2 to meet the high O_2 requirements of adult mammalian organs. A modification of the original grid method is widely used to study the growth and differentiation of adult and embryonic tissues.

sUses

Cultured organs can be an alternative for organs from other (living or deceased) people. This is useful as the availability of transplantable organs (derived from other people) is declining in developed countries. Another advantage is that

cultured organs, created using the patients own stem cell allows for organ transplants would allow the patient to no longer require immunosuppressive drugs.

Limitations

- Results from in vitro organ cultures are often not comparable to those from in vivo studies (e.g. studies on drug action) since the drugs are metabolized in vivo but not in vitro.

Cryopreservation

The process of storing or preserving the biological samples in extremely cold or subzero temperatures in a deep freeze; commonly at −196°C is termed as cryopreservation. The word "Cryopreservation" is derived from the Greek word "krúos", meaning icy cold or frost.

At such low temperatures, all the biological activities of the cells stop and the cell dies. Cryopreservation helps the cells to survive cooling to extreme temperatures and again thawing them to physiological conditions.

The ice formation inside the cells breaks the cell membrane and causes cell death. The freezing rate and the composition of the freezing medium are the two factors that can prevent this intercellular freezing.

In this process, biological materials including cells, tissues, organs, oocytes, spermatozoa, ovarian tissues, pre-implantation embryos, and other prepared culture media are preserved in extremely cold temperatures for extended periods without affecting the cell's viability.

Dry Ice and liquid nitrogen are generally used in this method of preservation.

At these subzero temperatures, all the biological activities of cells, tissues and other biological materials cease or effectively stopped and are presumed to provide indefinite longevity to cells.

Applications of Cryopreservation

Cryopreservation is a long-term storage technique, which is mainly used for preserving the biological material without decline or decaying the biological samples for an extended period of time at least for several thousands of years.

This method of preservation is widely used in different sectors including cryosurgery, molecular biology, ecology, food science, plant physiology, and in different medical applications. Other applications of cryopreservation process are:

1. Seed Bank.
2. Gene Bank.
3. Blood transfusion.
4. In vitro fertilization.
5. Organ transplantation.
6. Artificial insemination.
7. Freezing of cell cultures.
8. Storage of rare germplasm.
9. Conservation of biodiversity.
10. Conservation of endangered and disease free plant species.

Process of Cryopreservation

The complete procedure steps involved in preserving the obtained biological samples are as follows:

1. **Harvesting or Selection of material**– Few important criteria should be followed while selecting the biological materials such as – volume, density, pH, morphology, and damaged free.

2. **Addition of cry-protectant** – Cryoprotective agents such as glycerol, FBS, salts, sugars, glycols are added to the samples as it reduces the freezing point of the medium and also allow slower cooling rate, which reduces the risk of crystallization.

3. **Freezing** – Different methods of freezing are applied in this method of cryopreservation to protect cells from damage and cell death by their exposure to the warm solutions of cryoprotective agents.

4. **Storage in liquid nitrogen**– The cryopreserved samples are stored in extreme cold or -80°C in a freezer for at least 5 to 24 hours before transferring it to the storage vessels.

5. **Thawing-** The process of warming the biological samples in order to control the rate of cooling and prevent the cell damage caused by the crystallization.

Cryopreservation of Embryos

During the infertility treatment, hormones are used to stimulate the development of eggs. The eggs are then retrieved and fertilized in the lab. More embryos can be created to be transferred to the woman's uterus. These embryos can be cryopreserved and can be transferred at some later date. By this, the female can get an additional embryo transfer in future without spending on another IVF cycle.

Oocyte Cryopreservation

In the vitrification method, the eggs freeze rapidly so that there is little time for the ice crystals to form. New cryoprotectants are used that very high concentration of anti-freeze-like products.

The oocyte is first placed in a bath containing low concentration anti-freeze like cryoprotectant. Some sucrose is added to help draw some water out of the egg. The egg is then shifted to high concentration anti-freeze cryoprotectant for very few seconds and then immediately transferred to liquid nitrogen. When the egg has to be transplanted into the woman, the egg is thawed and used.

Cryopreservation of Sperm

The semen sample is mixed with a special solution to provide protection during freezing and thawing. The samples are then transferred to plastic vials and frozen in liquid nitrogen.

This process ensures the chances of conception in future. The sperm can also be deposited, froze and stored in cryobanks for less than a year. These sperms can later be used for certain infertility treatment procedures.

Benefits of Cryopreservation

There are numerous benefits of cryopreservation technique. These include:

- Fertility preservation.
- Minimal space and labour required.
- Safety from genetic contamination.
- Safeguards genetic integrity of valuable stains.
- Safeguards the germplasm of endangered species.
- Biological samples can be preserved for a longer period of time.
- Protects the samples from disease and microbial contamination.
- Prevents genetic drift by cryopreservation of gametes, embryos, etc

UNIT III

PRIMARY CULTURE

Primary culture is the cell culture system that is formed by culture cells directly obtained from tissue. A primary culture starts with the biopsy (~1 cm3) from tissue or organ via dissection. Thus, these cultures may contain a variety of differentiated cells e.g. fibroblasts, lymphocytes, macrophages, epithelial cells. Cell culture refers to the process by which cells are grown in a controlled artificial environment. Cells can be maintained in vitro outside of their original body by this process that is quite simple compared to organ and tissue culture.

In a cell culture technique, cells are removed from an animal or a plant, and grown subsequently in a favorable environment. For animal cell culture the cells are taken from the organ of an experimental animal. The cells may be removed directly or by mechanical or enzymatic action. The cells can also be obtained by previously made cell line or cell strain. Examples of cells used to culture are fibroblast, lymphocytes, cells from cardiac and skeletal tissues, cells from liver, breast, skin, and kidney and different types of tumor cells.

In tissue organization, cells have intercellular and cell basal membrane or cell matrix connections. For cells to be cultured, first of all, they need to get rid of these connections (single cell suspension). In the separation of cells, there are enzymatic or chemical digestion methods in which various proteolytic enzymes are used such as trypsin or collagenase and mechanic separation methods like splitting the tissue (mincing of the tissue) with surgical knives. Thus obtained cell suspensions are purified with serial dilution and centrifugation process and transferred into culture vessels. Then, many cells that are freed from cellular connections stick to the culture vessel. Primary culture process is completed once nonsticky cells and their residual are removed from the cell environment. However, except the cells that are initially wanted to be obtained in primary cultures, it is observed that different cell types especially fibroblasts also hold on to the culture vessel.

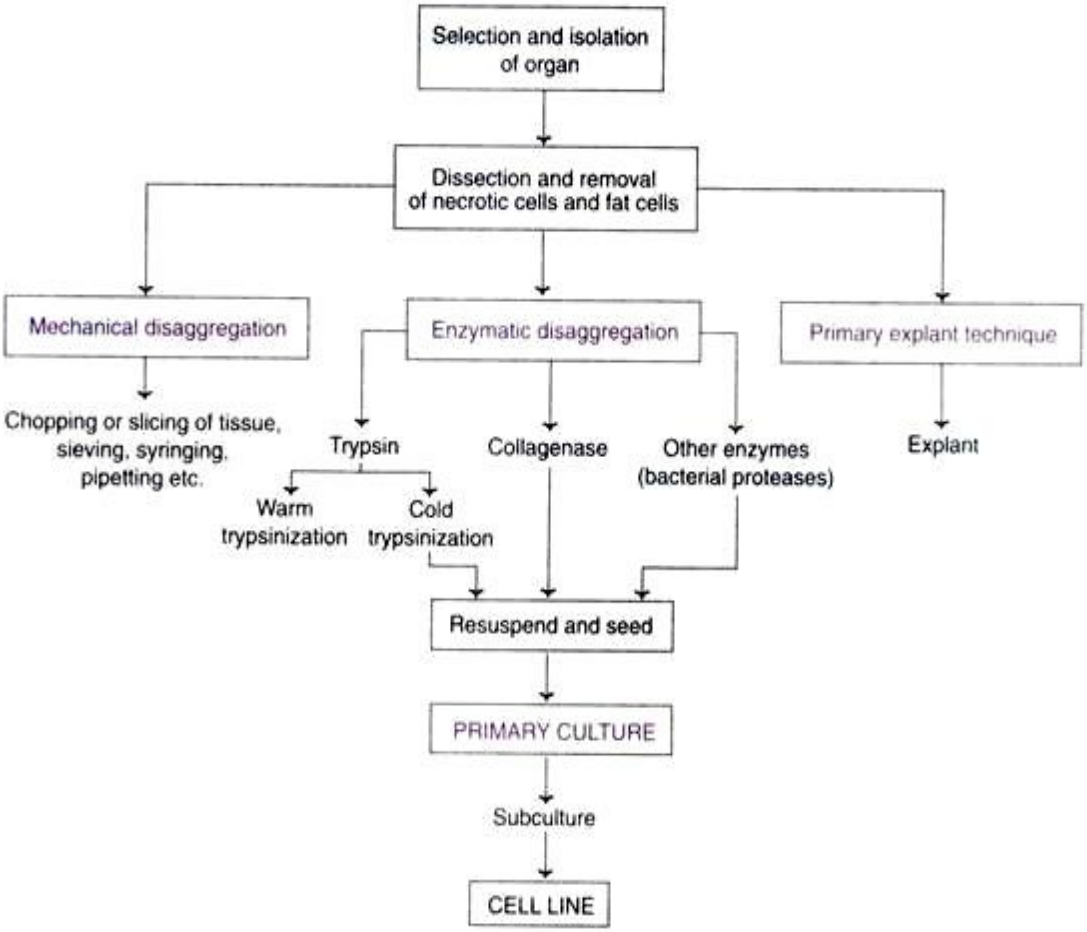


Fig. 36.1 : Different techniques used for primary culture.

LIMITATIONS:

When cells are taken out of the in vivo conditions and transferred into the culture environment, they might lose their structural and functional characteristics

Cells in the primary culture are quite significant in terms of being the closest forms of the state of the cells that they represent in normal tissues. Therefore, these cells should be reproduced with serial passages and store.

And in some other cases the number of cells that we are interested in might be less than other cells and that's why their reproduction might be pressed. In such a situation, intervention with special mediums or growth factors might be required.

DEVELOPMENT AND MAINTENANCE OF CELL LINES:

Growth Requirements

The culture media used for cell cultures are generally quite complex, and culture condition widely varies for each cell type. However, media generally include amino acids, vitamins, salts (maintain osmotic pressure), glucose, a bicarbonate buffer system (maintains a pH between 7.2 and 7.4), growth factors, hormones, O_2 and CO_2. To obtain best growth, addition of a small amount of blood serum is usually necessary, and several antibiotics, like penicillin and streptomycin are added to prevent bacterial contamination.

Temperature varies on the type of host cell. Most mammalian cells are maintained at 37^{o}C for optimal growth, while cells derived from cold- blooded animals tolerate a wider temperature range (i.e. 15^{o}C to 26^{o}C). Actively growing cells of log phage should be used which divide rapidly during culture.

Process to obtain primary cell culture

Primary cell cultures are prepared from fresh tissues. Pieces of tissues from the organ are removed aseptically; which are usually minced with a sharp sterile razor and dissociated by proteolytic enzymes (such as trypsin) that break apart the intercellular cement. The obtained cell suspension is then washed with a physiological buffer (to remove the proteolytic enzymes used). The cell suspension is spread out on the bottom of a flat surface, such as a bottle or a Petri dish. This thin layer of cells adhering to the glass or plastic dish is overlaid with a suitable culture medium and is incubated at a suitable temperature.

Aseptic techniques

Bacterial infections, like Mycoplasma and fungal infections commonly occur in cell culture creating a problem to identify and eliminate. Thus, all cell culture work is done in a sterile environment with proper aseptic techniques.

Work should be done in laminar flow with constant unidirectional flow of HEPA filtered air over the work area. All the material, solutions and the whole atmosphere should be of contamination-free.

Cryopreservation

If a surplus of cells is available from sub-culturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below –130°C until they are needed. This stores cell stocks and prevents original cell from being lost due to unexpected equipment failure or biological contaminations. It also prevents finite cells from reaching senescence and minimizes risks of changes in long term cultures.

When thawing the cells, the frozen tube of cells is warmed quickly in warm water, rinsed with medium and serum and then added into culture containers once suspended in the appropriate media.

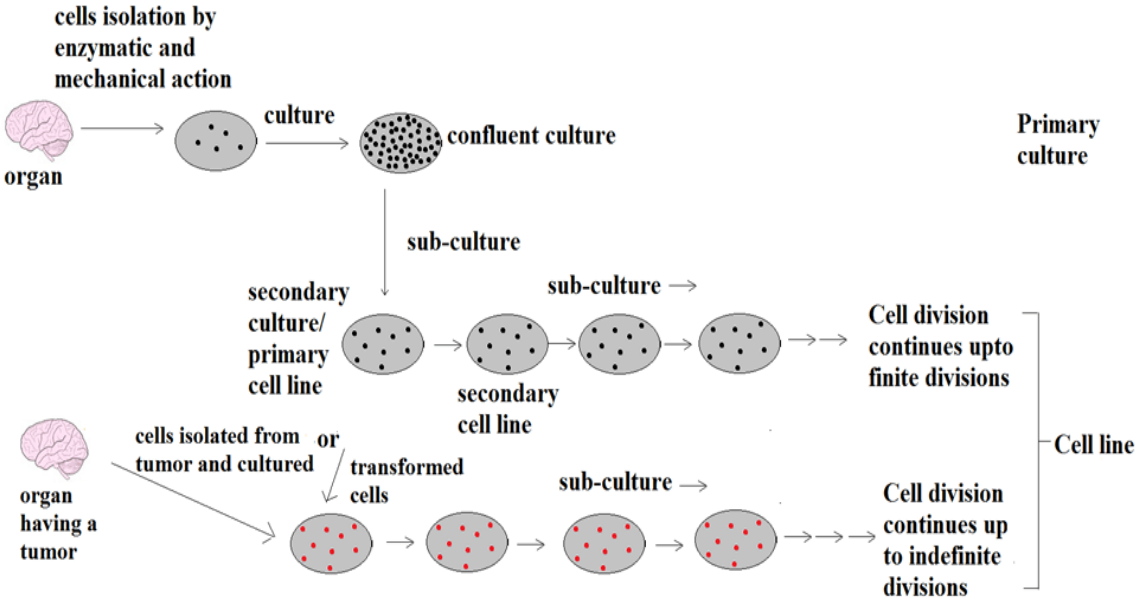


fig: animal cell culture

DISAGGREGATION-ENZYMATIC AND MECHANICAL

1. Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique.

2. Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin.

Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization (Fig. 36.2).

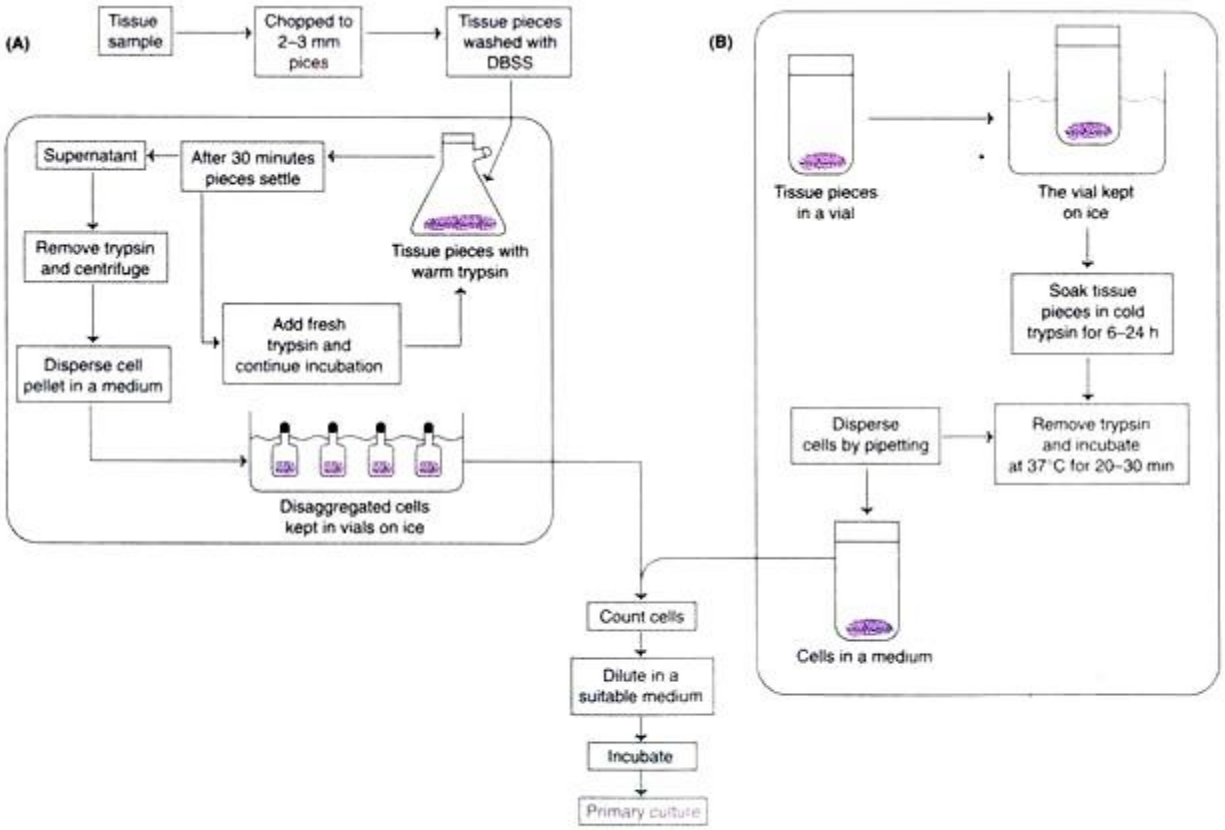


Fig. 36.2 : Preparation of primary culture by trypsin disaggregation (A) Warm trypsinization (B) Cold trypsinization (DBSS–Dissection basal salt solution).

Warm trypsinization:

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice).

The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

Cold trypsinization:

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique.

After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispesed by repeated pi-pettings. The dissociated cells can be counted, appropriately diluted and then used.

The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

Disaggregation by collagenase:

Collagen is the most abundant structural protein in higher animals. It is mainly present in the extra-cellular matrix of connective tissue and muscle. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the disaggregation of several tissues (normal or malignant) that may be sensitive to trypsin.

Highly purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase. The important stages in collagenase dis-aggregation, depicted in Fig. 36.3, are briefly described hereunder.

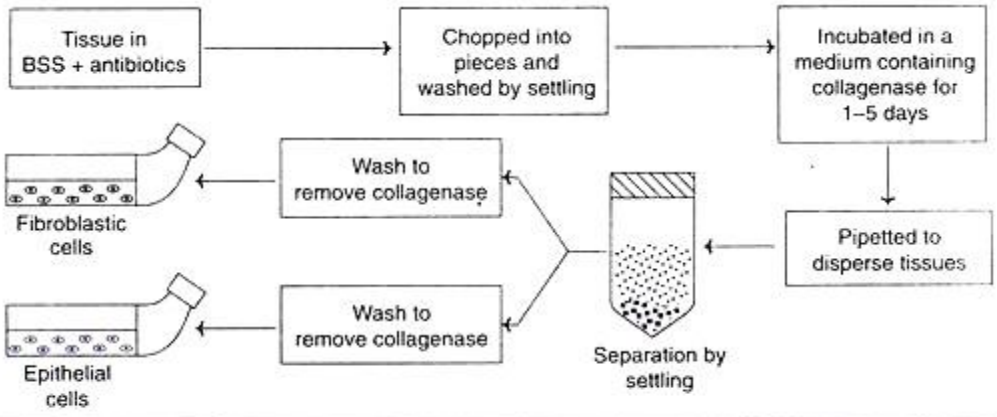


Fig. 36.3 : Important stages in collagenase disaggregation of tissue for primary culture (BSS–Basal salt solution).

The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated.

Collagenase disaggregation has been successfully used for human brain, lung and several other epithelial tissues, besides various human tumors, and other animal tissues. Addition of another enzyme hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation.

Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. Per-fusing the whole organ in situ can do this. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g. pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

MANIPULATION OF CELLS

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that rely on aseptic technique. Aseptic technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety cabinet or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g.amphotericin B and Antibiotic-Antimycotic solution) can also be added to the growth media.

As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium to measure nutrient depletion.

Media changes

In the case of adherent cultures, the media can be removed directly by

aspiration, and then is replaced. Media changes in non-adherent cultures involve centrifuging the culture and resuspending the cells in fresh media.

Passaging cells

Passaging (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA; however, other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture. Some cell cultures, such as RAW cells are mechanically scraped from the surface of their vessel with rubber scrapers.

Transfection and transduction

Another common method for manipulating cells involves the introduction of foreign DNA by transfection. This is often performed to cause cells to express a gene of interest. More recently, the transfection of RNAi constructs have been realized as a convenient mechanism for suppressing the expression of a particular gene/protein. DNA can also be inserted into cells using viruses, in methods referred to as transduction, infection or transformation. Viruses, as parasitic agents, are well suited to introducing DNA into cells, as this is a part of their normal course of reproduction.

ELECTROPORATION

Pulsed electrical fields can be used to introduce DNA into a wide variety of animal cells1,2. Electroporation works well with cell lines that are refractive to other techniques, such as calcium phosphate–DNA coprecipitation. But as with other transfection methods, the optimal conditions for electroporation of untested cell lines must be determined experimentally.

Molecules are suspended in a conductive solution, and an electrical circuit is closed around the mixture. An electrical pulse at an optimized voltage and only lasting a few microseconds to a millisecond is discharged through the cell suspension. This disturbs the phospholipid bilayer of the membrane and results in the formation of temporary pores. The electric potential across the cell membrane simultaneously rises to allow charged molecules like DNA to be driven across the membrane through the pores in a manner similar to electrophoresis.

The main advantage of electroporation is its applicability for transient and stable transfection of all cell types. Furthermore, because electroporation is easy and rapid, it is able to transfect a large number of cells in a short time once optimum electroporation conditions are determined. The major drawback of electroporation is substantial cell death caused by high voltage pulses and only partially successful membrane repair, requiring the use of greater quantities of cells compared to chemical transfection methods.

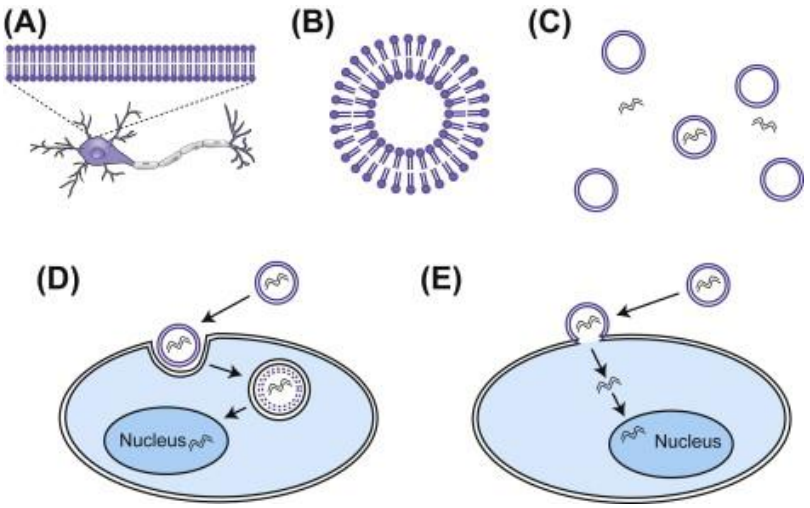
Electroporation Score Determination

For non-adherent cell lines, viability determination was based on trypan blue exclusion and/or determination of the % of cells displaying viable cell FSC vs. SSC parameters by flow cytometry analysis on cells negative after 7AAD staining. For adherent cells, viability determination was calculated based on the % of the OD obtained in Crystal Violet staining assays at d + 1 or d + 3. Calculation was based on the formula % = 100 × [OD for control (non-electroporated) cell line/(OD for control (non-electroporated) cell line + OD for electroporated cell line)]. The “electroporation score” was calculated based on cell viability (after normalization against the viability of non-transfected cells) and transgene expression on d + 1, and the score set to the formula “Viability (%)*Expression (%)/*F*.” A division factor (*F* = 50 for adherent cell lines and *F* = 100 for non-adherent cell lines) was used in the score formula to fit the results in the graph scale.

LIPOSOME MEDIATED TRANSFORMATION

Using liposomes to deliver DNA into different eukaryotic cell types results in higher efficiency and greater reproducibility than other transfection methods. In this unit, Basic Protocol the describes a transient expression system while an Alternate Protocol involves stable transformation and expression of DNA integrated into the genome of the transfected cell. In both protocols, plasmid DNA derived from either crude (miniprep) or purified (through CsCl) preparations is mixed with a liposome suspension comprised of cationic lipids and applied to monolayer cell cultures.

Lipofection, also known as “lipid transfection” or “liposome-based transfection,” uses a lipid complex to deliver DNA to cells. Lipids are a broad class of fat-soluble biomolecules, such as fats, oils, and waxes. The cell membranes of animal cells are composed of a bilayer of phospholipids with hydrophilic surfaces facing the cytoplasm and extracellular environment . Lipofection technology uses tiny vesicular structures called liposomes that have the same composition as the cell membrane . A scientist performs a simple reaction that forms a liposome around the DNA sequence to be transfected . Depending on the liposome and cell type, the liposome can be endocytosed or directly fuse with the cell membrane to release the DNA construct into cells



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(A) The cell membrane is composed of a lipid bilayer, with a hydrophobic interior and hydrophilic exterior. (B) Liposomes are also composed of a lipid bilayer arranged as a spherical shell. (C) A scientist performs a brief reaction that allows liposomes to form around DNA. (D) Cells in culture can endocytose the liposome, digesting it within vesicles to release DNA. (E) Alternatively, liposomes can directly fuse with the plasma membrane, directly releasing DNA into cells.

The advantage to lipofection is that it works in many cell types, including cultured neurons. Commercially available kits allow transfection reactions to be performed within 30 min and gene expression to be assayed within hours. However, like the calcium phosphate method, lipofection is almost exclusively used in cell culture experiments.

MICROINJECTION

Microinjection is an essential approach in the study of mammalian oocytes and early embryos, and is useful for the introduction of many molecules and reagents. When optimized, this approach allows for over 90%

oocyte survival, increasing confidence in experimental results.

View

Microinjection is a direct method to introduce DNA into either cytoplasm or nucleus. It is a microsurgical procedure conducted on a single cell, using a glass needle (i.e., a fine, glass microcapillary pipette), a precision positioning device (a micromanipulator) to control the movement of the micropipette, and a microinjector. Extrusion of fluid containing the genetic material through the micropipette uses hydrostatic pressure. Injections are typically carried out under direct visual control, using a microscope. The small tip diameters of these micropipettes, combined with the high precision of the micromanipulator, allow accurate and precise DNA delivery. Conceptually, microinjection is the simplest gene delivery method. However, it is difficult to apply. Although pronuclear injection of DNA is very efficient, it is a laborious procedure; only one cell at a time can be injected, typically allowing for only a few hundred cells to be transfected per experiment. The cytoplasmic injection of DNA has been observed to be less effective probably because of cytoplasmic degradation of DNA by cytoplasmic nuclease enzymes.

A more recent development after establishing the microinjection technique is developing capillary microinjection into cultured somatic cells growing on a solid support . This technique is now established as one of the most flexible technique for introducing DNA and even RNA into living cells. These developments have also helped researchers to study single cells for complicated cellular processes, structure, and functions *in vitro*. The microinjection of DNA by capillary injection into the cells is shown in Figure 3.3. Microinjection is still widely used to develop transgenic animals. One of the significant developments in the area of microinjection gene therapy is automated micromanipulations and microinjection processes as well as the control and standardization of cell preparation or the production of injection capillaries. Computer-assisted and microprocessor-controlled injection systems

have allowed high injection rates with reproducible results, thus allowing for quantitative microinjection. Along with nuclear microinjection of DNA, the microinjection technique has also been utilized for mitochondrial DNA delivery and cytoplast fusion for gene delivery to treat non-Mendelian genetic diseases caused by mitochondrial DNA mutations. In this fusion method, mitochondrial DNA in the cytoplast is transferred into mutant cells through cybrid formation. However, the injected plasmid DNA is rapidly degraded in the cytoplasm, with an apparent half-life of 50–90 min. hence, microinjecting naked DNA directly into the nucleus has shown to avoid cytoplasmic degradation and has shown much higher levels of gene expression than injection into the cytoplasm.

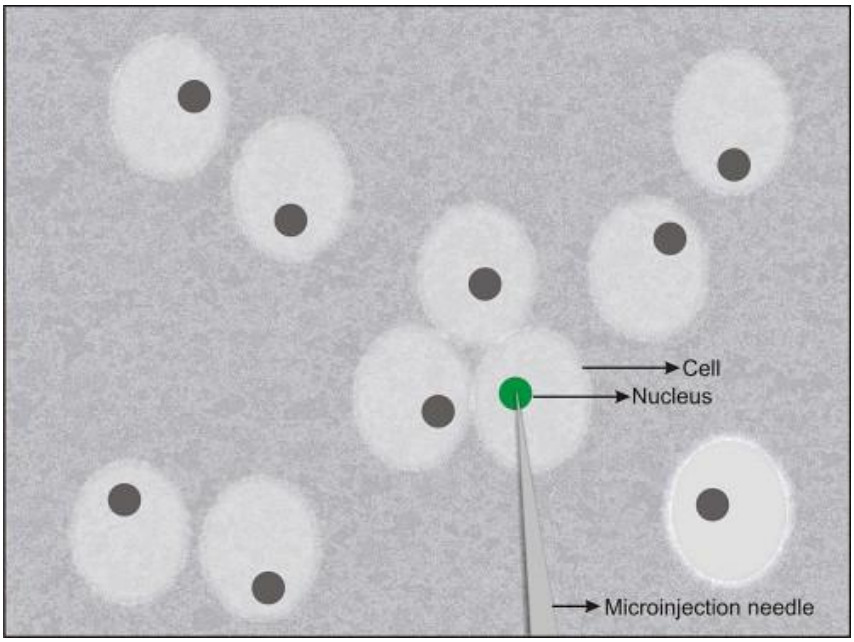


Figure: Microinjection of DNA into the nucleus of a single cell.

Microinjection has shown to deliver adenoviral vectors effectively in a controlled manner to tumors (xenotransplants) in nude mice and also provides reproducible transfection results . Microinjection has been very useful for producing recombinant cell lines; for example, green fluorescent protein (GFP) as a reporter gene effectively transferred to different cell lines like CHO DG44 and BHK-21 with satisfactory outcomes .

Even with the direct nuclear approach, microinjection is a laborious procedure. Only one cell can be injected at a time, and many injections are required before getting a successful transgene expression. Microinjection imposes a limitation on cell injections per experiment; so, with the current technology, microinjection has very limited applications for *in vivo* gene therapy.

SCALING- UP OF CELL LINES

Scale-up involves the development of culture systems in stages from (small scale) laboratory to (large scale) industry. The methodology adopted to increase the scale of a culture depends on the proliferation of cells and is broadly divided into two categories.

1. Scale-up in suspension.

2. Scale-up in monolayer.

Scale-Up in Suspension:

Scale-up in suspension is the preferred method as it is simpler. Scale-up of suspension culture primarily involves an increase in the volume of the culture. Small scale generally means the culture capacity less than 2 litres volume (or sometimes 5 litres).

Stirred suspension culture

It is usually necessary to maintain cell strains in stirred suspension cultures, by agitation (or stirring) of the medium. The stirring of the culture medium is achieved by a magnet encased in a glass pendulum or by a large surface area paddle. The stirring is usually done at a speed of 30-100 rpm. This is sufficient to prevent sedimentation of cells without creating shear forces that would damage cells.

Static suspension cultures:

Some cells can grow in suspension cultures, without stirring or agitation of the medium, and form monolayer cells. However, static suspension cultures are unsuitable for scale-up.

Factors in Scaling-Up:

For appropriate scale-up, the physical and chemical requirements of cells have to be satisfied.

Physical parameters:

i. Configuration of the bioreactor.

ii. Supply of power.

iii. Stirring of the medium.

Chemical parameters:

i. Medium and nutrients.

ii. Oxygen.

iii. pH and buffer systems.

iv. Removal of waste products.

Some of the relevant aspects of the factors in scale-up have already been described under cell culture-general considerations The most commonly used techniques for stirred suspension cultures are briefly described hereunder.

Stirrer Culture:

A diagrammatic representation of a stirrer flask is shown in Fig 37.2. The size of the stirrer flask is in the range of 2-10 litres. It is fitted with a magnetized rotating pendulum, and two side arms — one for the addition of cells and medium, and the other for the supply of CO2.

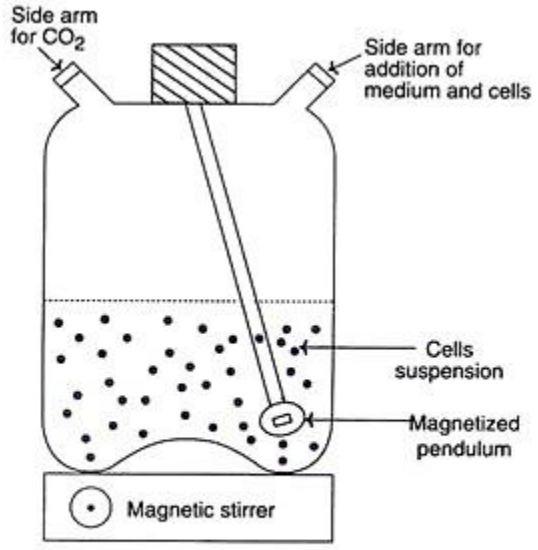


Fig. 37.2 : Diagrammatic representation of a stirrer flask.

The stirrer culture vessel is autoclaved (at 15 lb/ in2 for 15 minutes), and is then set up as in Fig. 37.2. The flask is seeded with the culture. Then medium along with an antifoam agent is added. The flask is connected to CO2 and stirred at a speed of 60 rpm. The flask is incubated for about 2 hours.

The contents of the small stirrer flask are transferred to a large flask and the entire set up is restarted. Incubation at 37°C is carried out for 4-7 days. The growth of the cells is monitored daily, and the cells are counted. There is a tendency of the cells to enter apoptosis, if the concentration exceeds 1 × 106 cells/ml.

Continuous Flow Culture:

In a continuous flow culture, it is possible to keep the cells at a desired and set concentration, and maintain. This is carried out by a bio-stat or chemo-stat (Fig 37.3).

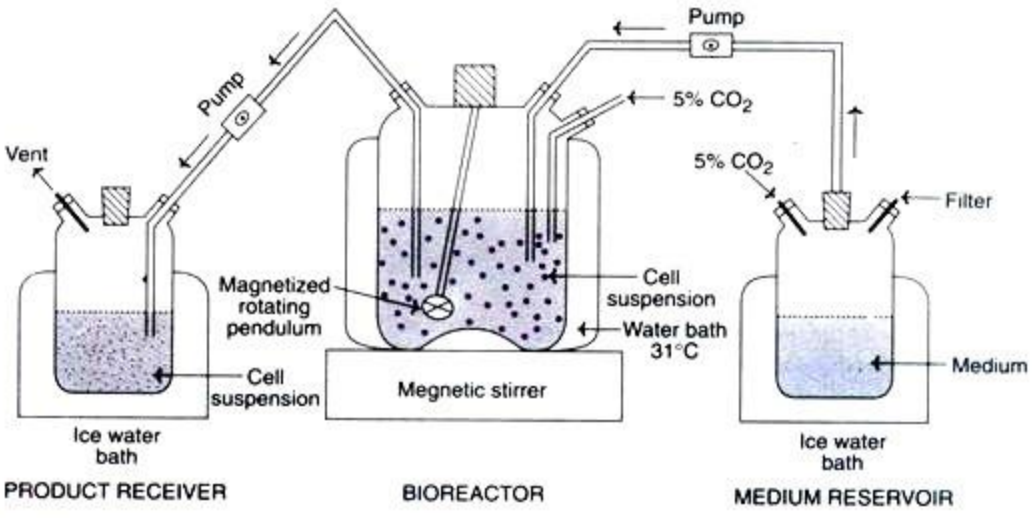


Fig. 37.3 : Suspension culture in a biostat (chemostat).

Continuous flow culture consists of growing the cells at the mid-log phase, removal of a measured volume of cells, and replacement by an equal volume of medium. The equipment, specially designed for this purpose has the facility for removal of the cells and addition of medium.

The flow rate of the medium addition can be determined from the growth rate of the culture. The medium flow can be regulated by a peristaltic pump. By this technique, it is possible to keep the culture conditions constant rather than to produce large number of cells. The continuous flow cultures are useful for monitoring metabolic changes in relation to cell density. However, these cultures are more susceptible to contamination.

Air-Lift Fermenter Culture:

The major limitation of scale-up in suspension culture is inadequate mixing and gas exchange. For small cultures, stirring of the medium is easy, but the problem is with large cultures. The design of fermenter should be such that maximum movement of liquid is achieved with minimum shear to damage the cells.

A diagrammatic representation of an air-lift fermenter is depicted in Fig. 37.4. A 5% CO2 in air is pumped through the bottom of the fermenter. The bubbles formed move up to agitate and aerate the culture. These bubbles carry a flow of liquid along with them and release at the top which goes to the bottom for recycling.

It is possible to continuously supply O2 to the culture in this technique. Air-lift fermenter culture technique is suitable for fragile animal as well as plant cells. This fermenter is extensively used in the biotechnology industry for culture capacities up to 20,000 litres.

NASA bioreactor:

NASA (National Aeronautics Space Administration, USA) constructed a bioreactor to grow the cells at zero gravity by slowly rotating the chamber (Fig. 37.5). The cells remain stationary and form three-dimensional aggregates and this enhances the product formation. In the NASA bioreactor, there is almost no shear force; hence the cells are not damaged.

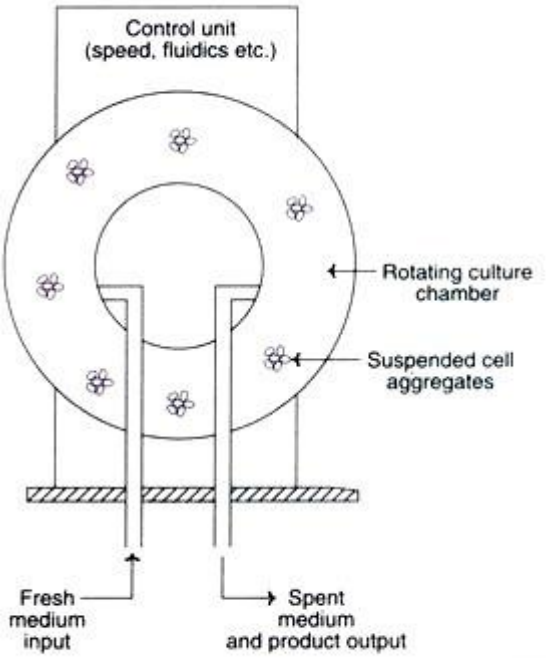


Fig. 37.5 : A bioreactor developed by NASA.

As the culture chamber stops its rotations, the cell aggregates sediment and the medium can be replaced.

Other Systems for Suspension Culture:

Rotating chambers:

The mixing and aeration of the culture medium can be achieved by 2 or 3 rotating chambers. The chambers are so designed that the cell suspension and mixing are high in one chamber while the product and spent medium remain in the other chamber. These chambers are separated by semipermeable membrane.

Perfused suspension culture:

This also has two compartments. The cells are kept in a low-volume compartment at high concentration, while the medium is perfused in adjacent compartment. The product can be collected in a third compartment.

Scale-Up in Monolayer:

The monolayer culture are anchorage- dependent. Therefore, for the scale-up of monolayer cultures, it is necessary to increase the surface area of the substrate in proportion to the number of cells and volume of the medium. Suspension cultures are preferred as they are simple. The advantages and disadvantages of monolayer cultures are listed.

Advantages:

i. Change of medium and washing of cells easy.

ii. It is easy to perfuse immobilized monolayer cells.

iii. The cell product formation (pharmaceutically important compounds e.g. interferon, antibodies) is much higher.

iv. The same set up and apparatus can be repeatedly used with different media and cells.

Disadvantages:

i. Tedious and costly.

ii. Require more space.

iii. Growth of cells cannot be monitored effectively.

iv. Difficult to measure control parameters (O2 pH, CO2 etc.)

For scale-up of monolayer cultures, a wide range of tissue cultures and system have been developed. A selected few of them are briefly described.

Roller Bottle Culture:

A round bottle or tube is rolled around its axis (by rollers) as the medium along with the cells runs around inside of the bottle (Fig. 37.6). As the cells are adhesive, they attach to inner surface of the bottle and grow forming a

monolayer.

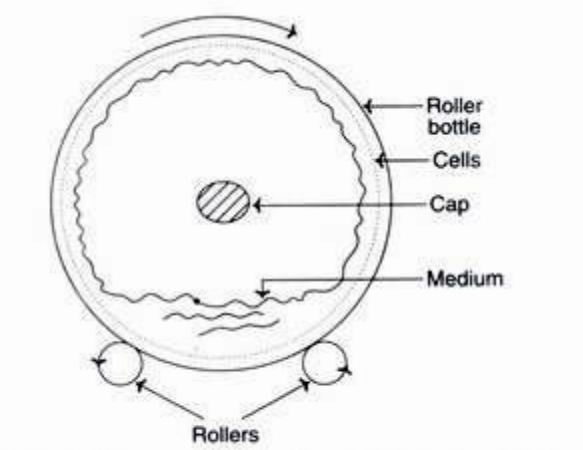


Fig. 37.6 : Roller bottle culture.

Roller bottle culture has certain advantages.

i. The medium is gently and constantly agitated.

ii. The surface area is high for cell growth.

iii. Collection of the supernatant medium is easy.

There are limitations in roller culture.

i. Monitoring of cells is very difficult.

ii. Investment is rather high.

Multi-surface Culture:

The most commonly used multi-surface propagator of monolayer is Slunclon cell factory (in short Nunc cell factory). It is composed of rectangular petri dish-like units with huge surface area (1,000-25,000 cm2). The units are inter-connected at two adjacent corners by vertical tubes (Fig. 37.7). The medium can flow between the compartments from one end.

The cell factory is almost like a conventional petridish or a flask with multiplayer units. The main limitation of cell factory is that it is very difficult to monitor the growth of cells. The major advantage however, is its simple operation to produce large number of cells.

Multi-array Disks, Spirals and Tubes:

The surface area for growth of monolayer cultures can be increased by using disks, spirals or tubes. They are however, not in common use as their commercial importance is limited.

Micro-carrier Culture:

Monolayers can be grown on small spherical carriers or micro-beads (80-300 pm diameter) referred to as micro-carriers. The micro-carriers are made up of any one the following materials (trade names given in brackets).

i. Plastic (acrobeads, bioplas).

ii. Glass (bioglass, ventreglas).

iii. Gelatin (ventregel, cytodex-3).

iv. Collagen (biospex, biospheres)

v. Cellulose (DE-52/53).

vi. DEAE Dextran (cytodex I, dormacell).

The micro-beads provide maximum surface area for monolayer cultures. This actually depends on the size and density of the beads. The cells can grow well on the smooth surface at the solid-liquid interface. However, micro-carriers need efficient stirring without grinding the beads. The main advantage with micro-carrier culture is that it can be treated as a suspension culture for all practical purposes.

Micro-carriers can be cultured in stirrer flask (See Fig. 37.2) or in continuous suspension (See Fig. 37.3). In fact, the suppliers of micro-carriers provide the technical literature and other relevant information for setting up a micro-carrier culture.

Factors affecting micro-carrier culture:

i. Composition and coating of beads (gelatin and collagen beads are preferred as they can be solubilized by proteases).

ii. Higher stirring speed is usually required.

iii. Glass beads are used when the micro-carriers need to be recycled.

Analysis of micro-carrier culture:

The cell counting techniques are difficult to be used for micro-carrier cultures. The growth rate can be detected by analyzing DNA or protein.

Perfused Monolayer Culture:

The growth surface areas of the monolayer cultures can be perfused to facilitate medium replacement and improved product formation and recovery. The perfusion can be carried out with pumps, oxygenator and other controllers. Perfusion of fixed and fluid-bed reactor is briefly described.

Fixed-bed reactors:

The fixed-bed reactor has a bed of glass beads (Fig. 37.8A). The medium is perfused upwards through the bed. The cells are grown on the surfaces of the beads. The products can be collected from the top along with the spent medium.

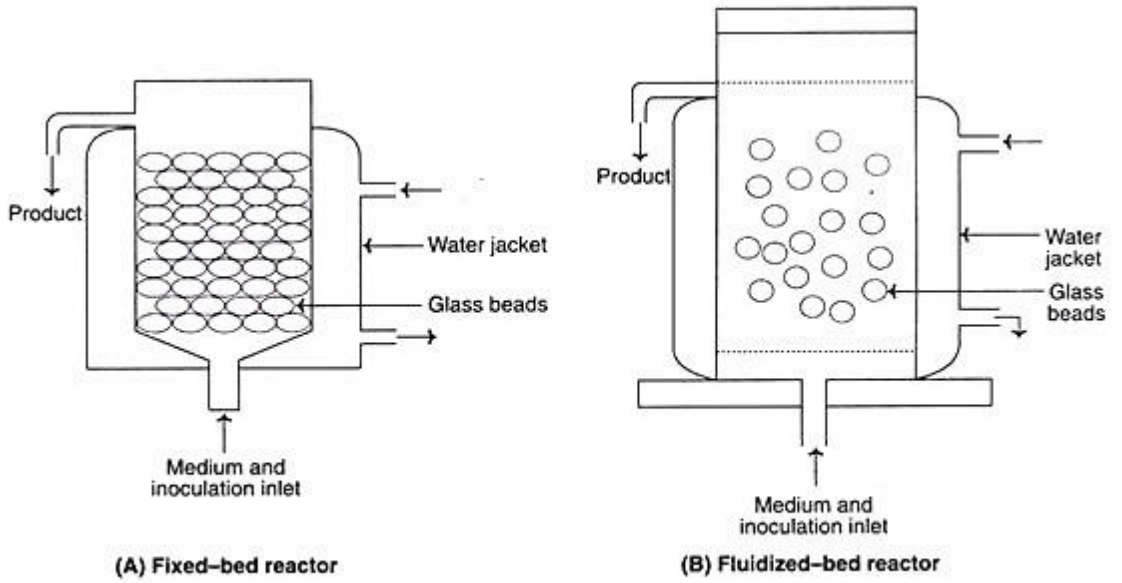


Fig. 37.8 : Fixed and fluidized–bed reactors.

Instead of glass beads, porous ceramic matrix with micro-channels can also be used in fixed-bed reactors.

Fluidized-bed reactors:

In a fluidized-bed reactor, the beads are suspended in a stream of medium (Fig. 37.8B). These beads are porous in nature, and are made up of ceramics or a mixture of ceramics mixed with natural products such as collagen. They are of low density and float in the medium. The flow rate of the perfused medium is equal to the sedimentation rate of the beads. The cells can grow as monolayers on the outer surfaces and inside of the porous beads.

Other perfused monolayer cultures:

Membrane perfusion, hollow-fiber perfusion, matrix perfusion and microencapsulation are among the other techniques for perfusion of monolayer cultures.

Monitoring of Cell Growth in Scale-Up:

Monitoring of the progress of cell growth and the culture systems are very important in scale-up.

Monitoring of Suspension Cultures:

The progress of suspension cultures can be monitored in situ by measuring glucose, O2, CO2, pH or metabolites produced (lactate, ammonia) or specialized products formed (e.g. immunoglobulin's by hybridoma cells).

The cell proliferation and rate of biomass formation can also be determined by estimating DNA, protein and ATP. The different parameters for monitoring of a bioreactor for suspension culture are depicted in Fig 37.9.

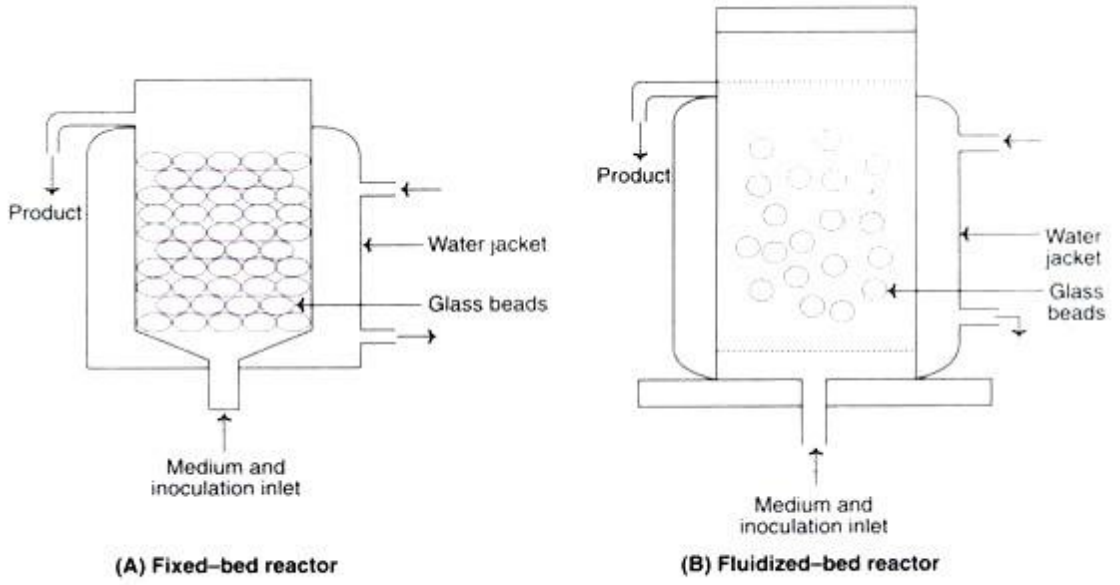


Fig. 37.8 : Fixed and fluidized–bed reactors.

Monitoring of Monolayer Cultures:

It is rather difficult to monitor monolayer cell cultures for scale-up. This is due to the fact that in most of the techniques employed for monolayer cultures, the cells cannot be observed directly to monitor the progress of the culture.

In recent years, nuclear magnetic resonance (NMR) technique is used to assay

the contents of culture. The characteristic NMR spectra generated by specific metabolites enables the identification and quantitation of metabolites, besides detecting the progress of cell growth.

CELL GROWTH, CHARACTERIZATION, CELL VIABILITY AND DEATH

Animal cells are more difficult to culture than microorganisms because they require many more nutrients and typically grow only when attached to specially coated surfaces. Despite these difficulties, various types of animal cells, including both undifferentiated and differentiated ones, can be cultured successfully.

Go to:

Rich Media Are Required for Culture of Animal Cells

Nine amino acids, referred to as the *essential amino acids,* cannot be synthesized by adult vertebrate animals and thus must be obtained from their diet. Animal cells grown in culture also must be supplied with these nine amino acids, namely, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition, most cultured cells require cysteine, glutamine, and tyrosine. In the intact animal, these three amino acids are synthesized by specialized cells; for example, liver cells make tyrosine from phenylalanine, and both liver and kidney cells can make glutamine. Animal cells both within the organism and in culture can synthesize the 8 remaining amino acids; thus these amino acids need not be present in the diet or culture medium. The other essential components of a medium for culturing animal cells are vitamins, which the cells cannot make at all or in adequate amounts; various salts; glucose; and *serum,* the noncellular part of the blood

Growth Media for Mammalian Cells.

Serum, a mixture of hundreds of proteins, contains various factors needed for

proliferation of cells in culture. For example, it contains insulin, a hormone required for growth of many cultured vertebrate cells, and transferrin, an iron-transporting protein essential for incorporation of iron by cells in culture. Although many animal cells can grow in a serum-containing medium, such as Eagle's medium, certain cell types require specific protein growth factors that are not present in serum. For instance, precursors of red blood cells require the hormone erythropoietin, and T lymphocytes of the immune system require interleukin 2 (IL-2). These factors bind to receptor proteins that span the plasma membrane, signaling the cells to increase in size and mass and undergo cell division (Chapter 20). A few mammalian cell types can be grown in a completely defined, serum-free medium supplemented with trace minerals, specific protein growth factors, and other components

Most Cultured Animal Cells Grow Only on Special Solid Surfaces

Within the tissues of intact animals, most cells tightly contact and interact specifically with other cells via various cellular junctions. The cells also contact the extracellular matrix, a complex network of secreted proteins and carbohydrates that fills the spaces between cells .The matrix, whose constituents are secreted by cells themselves, helps bind the cells in tissues together; it also provides a lattice through which cells can move, particularly during the early stages of animal differentiation.

The extracellular matrices in various animal tissues consist of several common components: fibrous collagen proteins; hyaluronan (or hyaluronic acid), a large mucopolysaccharide; and covalently linked polysaccharides and proteins in the form of proteoglycans (mostly carbohydrate) and glycoproteins (mostly protein). However, the exact composition of the matrix in different tissues varies, reflecting the specialized function of a tissue. In connective tissue, for example, the major protein of the extracellular matrix is a type of collagen that forms insoluble fibers with a very high tensile strength. Fibroblasts, the principal cell type in connective tissue, secrete this type of collagen as well as the other

matrix components. Receptor proteins in the plasma membrane of cells bind various matrix elements, imparting strength and rigidity to tissues (see Figure 5-40).

The tendency of animal cells in vivo to interact with one another and with the surrounding extracellular matrix is mimicked in their growth in culture. Unlike bacterial and yeast cells, which can be grown in suspension, most cultured animal cells require a surface to grow on. Many types of cells can adhere to and grow on glass, or on specially treated plastics with negatively charged groups on the surface (e.g., SO32-). The cultured cells secrete collagens and other matrix components; these bind to the culture surface and function as a bridge between it and the cells. Cells cultured from single cells on a glass or a plastic dish form visible colonies in 10 – 14 days (Figure 6-3). Some tumor cells can be grown in suspension, a considerable experimental advantage because equivalent samples are easier to obtain from suspension cultures than from colonies grown in a dish.

Classification of cytotoxicity and cell viability assays

Although there are different classifications for cytotoxicity and cell viability assays, in this chapter, these assays are classified according to measurement types of end points (color changes, fluorescence, luminescent etc.).

Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.
Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.
Fluorometric assays: alamarBlue assay and CFDA-AM assay.
Luminometric assays: ATP assay and real-time viability assay.

CYTOTOXICITY OF CULTURED CELLS

Cytotoxicity is one of the most important indicators for biological evaluation in vitro studies. In vitro, chemicals such as drugs and pesticides

have different cytotoxicity mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. In order to determine the cell death caused by these damages, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays. Cytotoxicity and cell viability assays are based on various cell functions. A broad spectrum of cytotoxicity assays is currently used in the fields of toxicology and pharmacology.

There are different classifications for these assays:

(i) Dye exclusion assays

(ii) Colorimetric assays

(iii) Fluorometric assays

(iv) Luminometric assays.

Choosing the appropriate method among these assays is important for obtaining accurate and reliable results. When selecting the cytotoxicity and cell viability assays to be used in the study, different parameters have to be considered such as the availability in the laboratory where the study is to be performed, test compounds, detection mechanism, specificity, and sensitivity. In this chapter, information will be given about in vitro cytotoxicity and viability assays, these assays will be classified and their advantages and disadvantages will be emphasized. The aim of this chapter is to guide the researcher interested in this subject to select the appropriate assay for their study.

Viability levels and/or proliferation rates of cells are good indicators of cell health. Physical and chemical agents can affect cell health and metabolism. These agents may cause toxicity on cells via different mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors, inhibition of polydeoxynucleotide elongation, and

enzymatic reactions [1]. In order to determine the cell death caused by these mechanisms, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays.

In vitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Application of these assays has been of increasing interest over recent years. Currently, these assays are also used in oncological researches to evaluate both compound toxicity and tumor cell growth inhibition during drug development. Because, they are rapid, inexpensive and do not require the use of animals. Furthermore, they are useful for testing large number of samples. Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity.

In vitro cytotoxicity and/or cell viability assays have some advantages, such as speed, reduced cost and potential for automation, and tests using human cells may be more relevant than some in vivo animal tests. However, they have some disadvantages because they are not technically advanced enough yet, to replace animal tests.

It is important to know how many viable cells are remaining and/or how many cells are dead at the end of the experiment. A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. The choice of assay method is crucial in the assessment of the interaction type.

UNIT IV

GENETIC ENGINEERING IN ANIMALS

METHODS FOR DNA TRANSFER:

CALCIUM PHOSPHATE CO-PRECIPITATION:

The introduction of DNA plasmids into cells is necessary for the study of various aspects of neuronal cell biology, including the investigation of gene and protein function by overexpression or knockdown, tracking of expressed proteins to subcellular compartments, and the study of protein turnover. Since no transfection method is suitable for all applications and cell types, researchers tend to use techniques suitable for specific applications and concerns, such as transfection efficiency, cell survival, expression levels, and applicability to experiments. There are four different categories of transfection methods: electrical transfection, chemical transfection, virus-based transfection, and physical transfection.

Chemical transfection methods, such as calcium phosphate coprecipitation and lipofection, are relatively simple to perform and do not require any specialized equipment. These advantages allow this method to be used widely in many cell types. Calcium phosphate transfection results in DNA crystals that are complexed with the calcium ions in the phosphate buffer. These crystals precipitate onto the cells and are presumably taken up by endocytosis. Although it is very easy and inexpensive, the calcium phosphate transfection method has not been used very often for primary neuronal cultures because it induces neuronal toxicity. However, when optimized, this method results in relatively low toxicity and high transfection efficiencies. This neuronal gene delivery can be accomplished using standard eukaryotic expression vectors.

Co-transfection of several plasmids and many different gene combinations can be applied. In addition, except at very early stages, neurons

at almost all stages of differentiation in culture can be transfected using this method. The time course and levels of protein expression can be easily manipulated by altering the amount of plasmid used. This method can also be used with a number of experimental procedures and treatments. This method is suitable for experiments requiring a relatively low number of transfected cells, such as those investigating the overexpression or knockdown effect of a gene of interest by imaging neuronal morphology of single cells in a neuronal network, which requires the identification of dendrites and axons from individual neurons of interest. Moreover, subcellular localization, colocalization, and trafficking studies of proteins can be performed.

Microinjection

Microinjection is the use of a glass micropipette to inject a liquid substance at a microscopic or borderline macroscopic level. The target is often a living cell but may also include intercellular space. Microinjection is a simple mechanical process usually involving an inverted microscope with a magnification power of around 200x (though sometimes it is performed using a dissecting stereo microscope at 40–50x or a traditional compound upright microscope at similar power to an inverted model).

For processes such as cellular or pronuclear injection the target cell is positioned under the microscope and two micromanipulators—one holding the pipette and one holding a microcapillary needle usually between 0.5 and 5 μm in diameter (larger if injecting stem cells into an embryo)—are used to penetrate the cell membrane and/or the nuclear envelope.[1] In this way the process can be used to introduce a vector into a single cell. Microinjection can also be used in the cloning of organisms, in the study of cell biology and viruses, and for treating male sub fertility through intra cytoplasmic sperm injection.

History

The use of microinjection as a biological procedure began in the early twentieth century, although even through the 1970s it was not commonly used. By the 1990s, its use had escalated significantly and it is now considered

common laboratory technique, along with vesicle fusion, electroporation, chemical transfection, and viral transduction, for introducing a small amount of a substance into a small target

Basic types

There are two basic types of microinjection systems. The first is called a *constant flow system* and the second is called a *pulsed flow system*. In a constant flow system, which is relatively simple and inexpensive though clumsy and outdated, a constant flow of a sample is delivered from a micropipette and the amount of the sample which is injected is determined by how long the needle remains in the cell. This system typically requires a regulated pressure source, a capillary holder, and either a coarse or a fine micromanipulator. A pulsed flow system, however, allows for greater control and consistency over the amount of sample injected: the most common arrangement for intracytoplasmic sperm injection includes an Eppendorf "Femtojet" injector coupled with an Eppendorf "InjectMan", though procedures involving other targets usually take advantage of much less expensive equipment of similar capability. Because of its increased control over needle placement and movement and in addition to the increased precision over the volume of substance delivered, the pulsed flow technique usually results in less damage to the receiving cell than the constant flow technique. However, the Eppendorf line, at least, has a complex user interface and its particular system components are usually much more expensive than those necessary to create a constant flow system or than other pulsed flow injection systems

Pronuclear injection

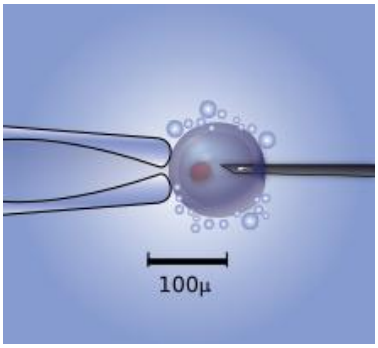


Diagram of the intracytoplasmic sperm injection of a human egg. Micromanipulator on the left holds egg in position while microinjector on the right delivers a single sperm cell.

Pronuclear injection is a technique used to create transgenic organisms by injecting genetic material into the nucleus of a fertilized oocyte. This technique is commonly used to study the role of genes using mouse animal models.

Pronuclear injection in mice

The pronuclear injection of mouse sperm is one of the two most common methods for producing transgenic animals (along with the genetic engineering of embryonic stem cells). In order for pronuclear injection to be successful, the genetic material (typically linear DNA) must be injected while the genetic material from the oocyte and sperm are separate (i.e., the pronuclear phase). In order to obtain these oocytes, mice are commonly superovulated using gonadotrophins.

Once plugging has ocurred, oocytes are harvested from the mouse and injected with the genetic material. The oocyte is then implanted in the oviduct of a pseudopregnant animal. While efficiency varies, 10-40% of mice born from these implanted oocytes may contain the injected construct. Transgenic mice can then be bred to create transgenic lines.

Electroporation:

Electropermeabilization**,** is a microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (also called electrotransfer). In microbiology, the process of electroporation is often used to transform bacteria, yeast, or plant protoplasts by introducing new coding DNA. If bacteria and plasmids are mixed together, the plasmids can be transferred into the bacteria after electroporation, though depending on

what is being transferred cell-penetrating peptides or Cell Squeeze could also be used. Electroporation works by passing thousands of volts across a distance of one to two millimeters of suspended cells in an electroporation cuvette (1.0 – 1.5 kV, 250 – 750 V/cm).

Afterwards, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain reproduced plasmids. This process is approximately ten times more effective than chemical transformation.

Electroporation is also highly efficient for the introduction of foreign genes into tissue culture cells, especially mammalian cells.[4] For example, it is used in the process of producing knockout mice, as well as in tumor treatment, gene therapy, and cell-based therapy. The process of introducing foreign DNA into eukaryotic cells is known as transfection. Electroporation is highly effective for transfecting cells in suspension using electroporation cuvettes. Electroporation has proven efficient for use on tissues in vivo, for in utero applications as well as in ovo transfection. Adherent cells can also be transfected using electroporation, providing researchers with an alternative to trypsinizing their cells prior to transfection. One downside to electroporation, however, is that after the process the gene expression of over 7,000 genes can be affected. This can cause problems in studies where gene expression has to be controlled to ensure accurate and precise results.

Although bulk electroporation has many benefits over physical delivery methods such as microinjections and gene guns, it still has limitations including low cell viability. Miniaturization of electroporation has been studied leading to microelectroporation and nanotransfection of tissue utilizing electroporation based techniques via nanochannels to minimally invasively deliver cargo to the cells.

Cell fusion is of interest not only as an essential process in cell biology, but also as a useful method in biotechnology and medicine. Artificially induced fusion can be used to investigate and treat different diseases, like diabetes, regenerate axons of the central nerve system, and produce cells with

desired properties, such as in cell vaccines for cancer immunotherapy. However, the first and most known application of cell fusion is production of monoclonal antibodies in hybridoma technology, where hybrid cell lines (hybridomas) are formed by fusing specific antibody-producing B lymphocytes with a myeloma (B lymphocyte cancer) cell line.

Electroporation is performed with electroporators, purpose-built appliances which create an electrostatic field in a cell solution. The cell suspension is pipetted into a glass or plastic cuvette which has two aluminium electrodes on its sides. For bacterial electroporation, typically a suspension of around 50 microliters is used. Prior to electroporation, this suspension of bacteria is mixed with the plasmid to be transformed. The mixture is pipetted into the cuvette, the voltage and capacitance are set, and the cuvette is inserted into the electroporator. The process requires direct contact between the electrodes and the suspension. Immediately after electroporation, one milliliter of liquid medium is added to the bacteria (in the cuvette or in an Eppendorf tube), and the tube is incubated at the bacteria's optimal temperature for an hour or more to allow recovery of the cells and expression of the plasmid, followed by bacterial culture on agar plates.

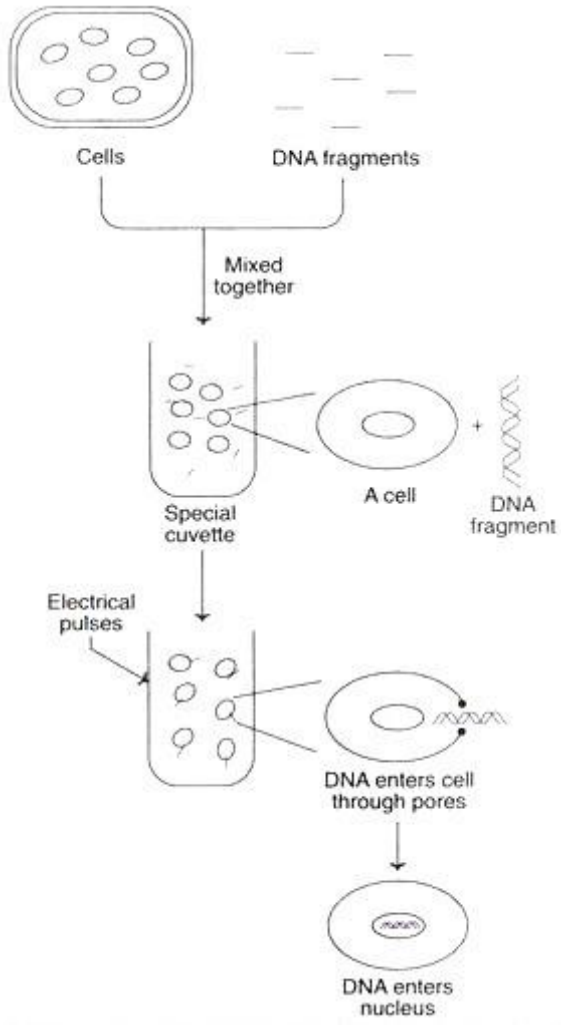
The success of the electroporation depends greatly on the purity of the plasmid solution, especially on its salt content. Solutions with high salt concentrations might cause an electrical discharge (known as arcing), which often reduces the viability of the bacteria. For a further detailed investigation of the process, more attention should be paid to the output impedance of the porator device and the input impedance of the cells suspension (e.g. salt content).

Since the cell membrane is not able to pass current (except in ion channels), it acts as an electrical capacitor. Subjecting membranes to a high-voltage electric field results in their temporary breakdown, resulting in pores that are large enough to allow macromolecules (such as DNA) to enter or leave the cell.

Additionally, electroporation can be used to increase permeability of cells during in Utero injections and surgeries. Particularly, the electroporation allows for a more efficient transfection of DNA, RNA, shRNA, and all nucleic acids into the cells of mice and rats. The success of in vivo electroporation depends greatly on voltage, repetition, pulses, and duration. Developing central nervous systems are most effective for in vivo electroporation due to the visibility of ventricles for injections of nucleic acids, as well as the increased permeability of dividing cells. Electroporation of injected in utero embryos is performed through the uterus wall, often with forceps-type electrodes to limit damage to the embryo.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

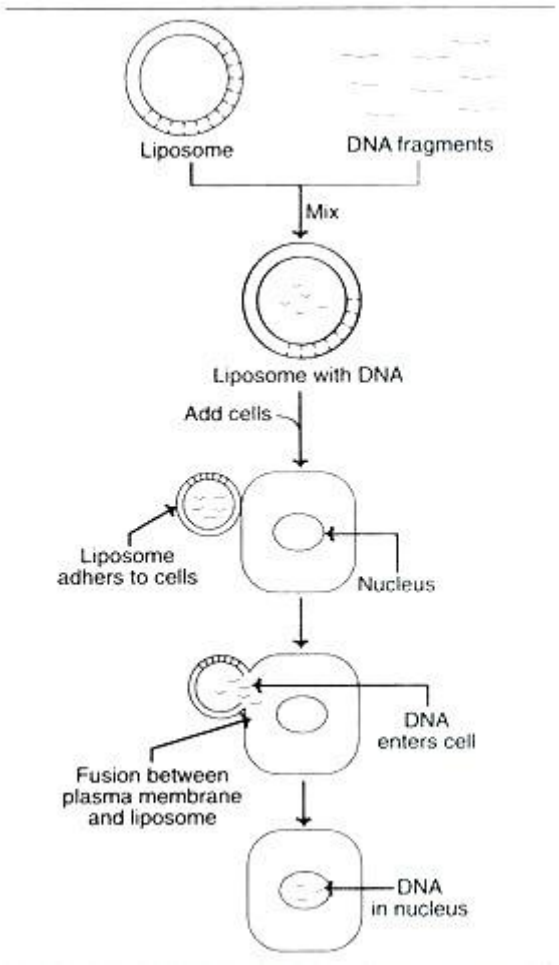
The basic technique of electroporation for transferring genes into mammalian cells is depicted in figure. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.



Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Liposome-Mediated Gene Transfer:

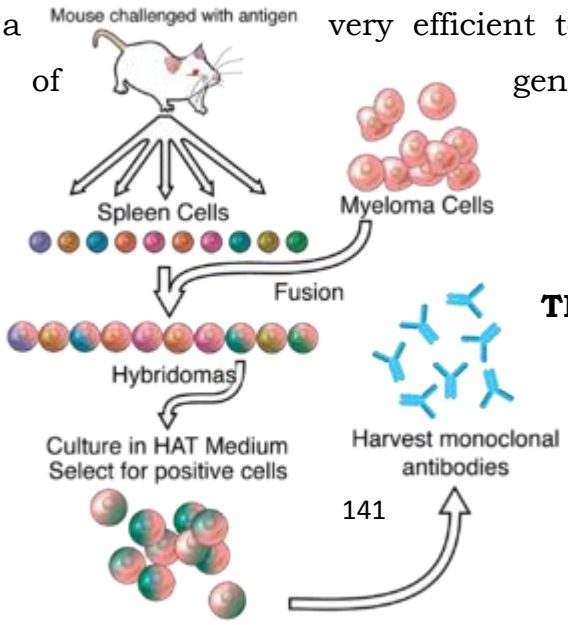
Liposomes are circular lipid molecules, which have an aqueous interior that can carry nucleic acids. Several techniques have been developed to encapsulate DNA in liposomes. The liposome- mediated gene transfer, referred to as lipofection, is depicted in Figure



On treatment of DNA fragment with liposomes, the DNA pieces get encapsulated inside liposomes. These liposomes can adher to cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. The positively charged liposomes very efficiently complex with DNA, bind to cells and transfer DNA rapidly.

Lipofection is a very efficient technique and is used for the transfer of genes to bacterial, animal and plant cells.

HYBRIDOMA TECHNOLOGY

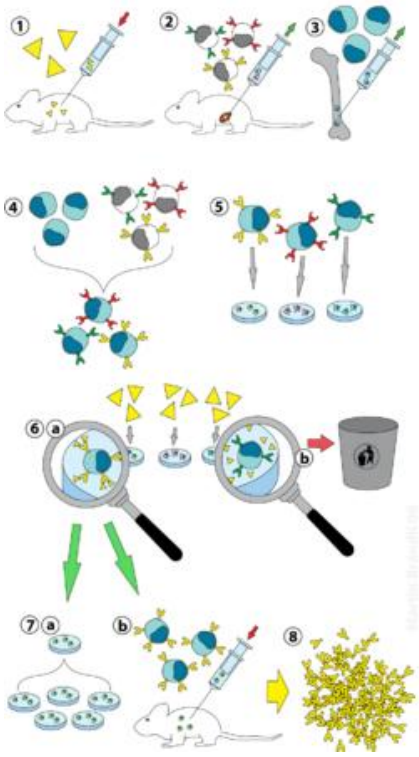


A general representation of the hybridoma method used to produce monoclonal antibodies.

Hybridoma technology is a method for producing large numbers of identical antibodies (also called monoclonal antibodies). This process starts by injecting a mouse (or other mammal) with an antigen that provokes an immune response. A type of white blood cell, the B cell, produces antibodies that bind to the injected antigen. These antibody producing B-cells are then harvested from the mouse and, in turn, fused with immortal B cell cancer cells, a myeloma, to produce a hybrid cell line called a hybridoma, which has both the antibody-producing ability of the B-cell and the longevity and reproductivity of the myeloma.

The hybridomas can be grown in culture, each culture starting with one viable hybridoma cell, producing cultures each of which consists of genetically identical hybridomas which produce one antibody per culture (monoclonal) rather than mixtures of different antibodies (polyclonal). The myeloma cell line that is used in this process is selected for its ability to grow in tissue culture and for an absence of antibody synthesis. In contrast to polyclonal antibodies, which are mixtures of many different antibody molecules, the monoclonal antibodies produced by each hybridoma line are all chemically identical.

The production of monoclonal antibodies was invented by César Milstein and Georges J. F. Köhler in 1975. They shared the Nobel Prize of 1984 for Medicine and Physiology with Niels Kaj Jerne, who made other contributions to immunology. The term hybridoma was coined by Leonard Herzenberg during his sabbatical in César Milstein's laboratory in 1976–1977.



(1) Immunisation of a mouse
(2) Isolation of B cells from the spleen
(3) Cultivation of myeloma cells
(4) Fusion of myeloma and B cells
(5) Separation of cell lines
(6) Screening of suitable cell lines
(7) *in vitro* **(a)** or *in vivo* **(b)** multiplication
(8) Harvesting

Laboratory animals (mammals, e.g. mice) are first exposed to the antigen that an antibody is to be generated against. Usually this is done by a series of injections of the antigen in question, over the course of several weeks. These injections are typically followed by the use of in vivo electroporation, which significantly enhances the immune response. Once splenocytes are isolated from the mammal's spleen, the B cells are fused with immortalised myeloma cells. The fusion of the B cells with myeloma cells can be done using

electrofusion. Electrofusion causes the B cells and myeloma cells to align and fuse with the application of an electric field.

Alternatively, the B-cells and myelomas can be made to fuse by chemical protocols, most often using polyethylene glycol. The myeloma cells are selected beforehand to ensure they are not secreting antibody themselves and that they lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene, making them sensitive to the HAT medium (see below).

Fused cells are incubated in HAT medium (hypoxanthine-aminopterin-thymidine medium) for roughly 10 to 14 days. Aminopterin blocks the pathway that allows for nucleotide synthesis. Hence, unfused myeloma cells die, as they cannot produce nucleotides by the *de novo* or salvage pathways because they lack HGPRT. Removal of the unfused myeloma cells is necessary because they have the potential to outgrow other cells, especially weakly established hybridomas. Unfused B cells die as they have a short life span. In this way, only the B cell-myeloma hybrids survive, since the HGPRT gene coming from the B cells is functional. These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells). The incubated medium is then diluted into multi-well plates to such an extent that each well contains only one cell. Since the antibodies in a well are produced by the same B cell, they will be directed towards the same epitope, and are thus monoclonal antibodies.

The next stage is a rapid primary screening process, which identifies and selects only those hybridomas that produce antibodies of appropriate specificity. The first screening technique used is called ELISA. The hybridoma culture supernatant, secondary enzyme labeled conjugate, and chromogenic substrate, are then incubated, and the formation of a colored product indicates a positive hybridoma. Alternatively, immunocytochemical screening can also be used.

The B cell that produces the desired antibodies can be cloned to produce many identical daughter clones. Supplemental media containing interleukin-6 (such as briclone) are essential for this step. Once a hybridoma colony is

established, it will continually grow in culture medium like RPMI-1640 (with antibiotics and fetal bovine serum) and produce antibodies.

Multiwell plates are used initially to grow the hybridomas, and after selection, are changed to larger tissue culture flasks. This maintains the well-being of the hybridomas and provides enough cells for cryopreservation and supernatant for subsequent investigations. The culture supernatant can yield 1 to 60 μg/ml of monoclonal antibody, which is maintained at -20 °C or lower until required.

By using culture supernatant or a purified immunoglobulin preparation, further analysis of a potential monoclonal antibody producing hybridoma can be made in terms of reactivity, specificity, and cross-reactivity.

Applications

The use of monoclonal antibodies is numerous and includes the prevention, diagnosis, and treatment of disease. For example, monoclonal antibodies can distinguish subsets of B cells and T cells, which is helpful in identifying different types of leukaemias. In addition, specific monoclonal antibodies have been used to define cell surface markers on white blood cells and other cell types. This led to the cluster of differentiation series of markers. These are often referred to as CD markers and define several hundred different cell surface components of cells, each specified by binding of a particular monoclonal antibody. Such antibodies are extremely useful for fluorescence-activated cell sorting, the specific isolation of particular types of cell

BIOLOGICAL VECTORS

All living vectors, including flies, roaches, mice, rats, etc. can transmit disease. They can be either passive or active

Passive Biologic Vectors

Passive biologic vectors are not as dangerous because they carry a smaller load of pathogens, and pathogens show decreased virulence due to

unfavorable growth conditions. For example, flies carry E. coli and Salmonella from feces by landing on them, but the organisms do not grow on the fly.

Active Biologic Vectors

Active biologic vectors have the infectious pathogen growing in them. They have a larger load and higher virulence because the pathogen can strive inside the host.

For instance, the malaria parasite has a life cycle inside the mosquito and can better infect a human host because it was allowed to grow.

Another example of an active biologic vector is any animal that has rabies. Rabies can grow in humans, but humans are not considered vectors because they do not transmit the disease (biting).

VACCINE PRODUCTION:

Introduction

Vaccination is one of the most successful and cost-effective public health interventions of all. It has eradicated smallpox, lowered the global incidence of polio by 99% since 1988 and achieved dramatic reductions in diseases such as measles, diphtheria, whooping cough (pertussis), tetanus and hepatitis B. *"Vaccination is the 20th century's greatest public health triumph after improved access to clean, safe drinking water."* (1)

Prior to the advent of cell culture, viruses could be propagated only on whole organisms, animal or plants. Whole organisms could include the natural host and laboratory animals such as chicken embryonated eggs, rabbits, mice, rats and others. The development of cell culture techniques in the 1950's opened the door to the manufacturing of a wide range of biological pharmaceutical products at industrial scale.

Definitions

Tissue culture is the general term for the removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial medium environment for maintaining cell viability.

The culture of whole organs or intact organ fragments with the intent to use cells as machinery to produce biological is called **Organ Culture**.

When the cells are removed from the organ fragments prior to or during cultivation thus disrupting their normal relationships with neighboring cell, the technology is called **Cell Culture**.

When cells are individually dissociated from an organism and placed into a suitable medium and support culture environment, they will attach, divide and grow. This cell culture is named **Primary Culture**. Cell culture may be initiated from normal, embryonic or malignant tissue. When cells in the primary culture vessel have grown and filled up all of the available culture substrate, they may be sub-cultured and give place for continued growth.

Following serial sub-culture of primary cells, cells that continue to grow are subjected to genetic reorganization and adapt to the new synthetic environment and proliferate serially, generating an immortalization event that results in a **Continuous Cell Line** over several passages or definitely immortal cells. Cells used for vaccine production, and acceptability.

PrimaryCells

Primary cell cultures (PCCs) can be established from safe animals or embryos, or from selected tissues from embryos, new born animals, or adult animals of almost any species. Primary cells do not expand much, but can be dissociated with protease and expanded in a new container for multiplication. The first requirements for cell substrates were published by the World Health Organization (WHO) in 1959 for the production of IPV vaccine derived from the kidney of clinically healthy monkeys. Those requirements were revised and re-published in 1966. The use of animals bred in a carefully controlled colony especially those that are specific pathogen free are strongly recommended.

Diploïdcelllines

The next major event in the cell substrate area was the development of human diploid cell (HDCs) in 1960s, proposed as an alternative to primary monkey kidney cells. Dr Leonard Hayflick developed a number of cell strains from normal human embryonic tissues. Acceptance of HDCs was based on a

significant amount of characterization data showing a normal chromosomal constitution, ability to generate cryo-preserved banks, a finite lifespan and inability to form tumors in animal models. With such characterization and leadership, the diploid cell strain concept was accepted in Europe and several National Health Authorities approved

Hayflick's WI-38 cell line for vaccines use and several years later also the MRC-5 cell line.

Based on the experience gained with HDCs, the characterization of cells became a central feature in the evaluation of all new cell types. The United States initially maintained its opposition based mostly on a speculative fear that diploid cell strains might harbor a human leukemia virus, but only until 1972, when the US Regulatory Agency approved the use of WI-38 cell line for oral live polio vaccine production, and a license was granted for live rubella virus vaccine in 1977.

Continuous Cell Lines

Normal vertebrate cells cannot be passed indefinitely in culture. After a limited number of cell doublings depending on the age and species of the original tissue, cultured cells stop to divide and then degenerate and die in a phenomenon called "crisis" or senescence. At anytime during the culture cells may become transformed meaning that they are no longer subject to crisis and senescence but can be sub-cultured for an undefined period of time. The transformation is a complex phenomenon in which cells become immortalized and are named "cell line" or "continuous cell line" in which case cells can be indefinitely propagated by subculturing.

Several techniques can be used to obtain an immortalized cellular lineage from primary cells:

1. Carry on passages of a normal cell culture and obtain continuous cell line, often described as spontaneous transformation (e.g., Vero, BHK-21, CHO, MDCK)
2. Immortalization can be induced by treatment with chemical mutagens (e.g., QT35, LMH),

3. Hybridization between host cell with an immortalized cell line (e.g., hybridoma),
4. Transfection with ectopic expressed genes involved in the cell cycle, such as E1 adenovirus (e.g., HEK-293, PER-C6),
5. Transfection with hTERT telomerase gene to enable indefinite replication of normal cells.

Under particular conditions, certain cells are able to spontaneously transform into immortal cell lines. The mechanisms by which cells gain immortalization are not fully understood, although a general requirement for oncoproteins such as human papillomavirus E6 and E7 has suggested that the p53 and Rb pathways are targeted. Genetic modifications induced during the immortalization process can have consequences on the karyotype with cells becoming aneuploid and containing abnormalities in chromosome number and structure.

Continuous cell lines (CCLs) have been used for the production of safe and effective bio-therapeutics and vaccines since the 1970's. To date a limited number of cell lines have been authorized by Health Authorities to produce vaccines, limited to CCLs obtained from normal tissues.

The Vero cell line, which was the first continuous mammalian cell line established from African green monkeys in 1962, is currently the most widely accepted by regulatory authorities for vaccine manufacturing. This is due to the fact that Vero-derived human vaccines have been used for nearly 40 years. The Vero cells can be grown and infected on micro-carrier beads in large-scale fermentors and in serum-free medium with no loss in productivity.

A WHO Expert Committee and the International Alliance for Biological Standardization (IABS) concluded in 1996 that the presence of 10 ng of heterogeneous DNA of non tumorigenic CCL per dose of product poses negligible risk for safety and that "nuclease treatment of products during manufacture would probably add more concerns than it would remove." The group concluded that every product must stand on its own merits for safety and efficacy and that the use of alternative manufacturing methods not

involving the use of continuous cell lines would be irrelevant to the acceptance or rejection of a product derived from a continuous cell line.

The next step of cell acceptability, for therapeutic drug, was in 1970s when interferon (IFN), found to have an interesting therapeutic potential, had to be produced at large scale. At that time the only source of IFN was from primary human lymphocytes which had a very limited productivity. This issue could be solved by the use of human lymphoma cells (Namalva cell line). However, Namalva cells contain an integrated Epstein Barr virus and unrelated cancer-sequences. After considerable discussion there was agreement from the regulatory agencies in the US and Europe to allow the use of Namalva cells for the production of IFN. Nevertheless, all possible functional DNA would need to be eliminated from the biological product with DNAses treatment and purification steps during the manufacturing process.

Monoclonal antibody technology, through development of recombinant DNA, facilitated the development of continuous cell lines that could secrete antibody of defined specificity.

The first tumorigenic cell line to be considered for use in the production of a live viral vaccines was the PER.C6 cell line. It was used for the development and production of a replication-defective adenovirus vectored HIV-1 vaccine (VRBPAC 2001). While adventitious agents and residual cell-substrate DNA are potential concerns with all novel cell substrates, there may be a heightened concern when the cell substrate is tumorigenic or derived from a tumor. In particular, the potential risk of adventitious agent contamination (including TSE contamination, since PER.C6 cells are neural derived), of residual cell-substrate DNA, and of whole cells were considered.

The next tumorigenic cell line under consideration was the Madin-Darby canine kidney (MDCK) cell line, proposed for the production of inactivated influenza virus vaccines (VRBPAC 2005). Additionally, a non-tumorigenic MDCK cell line was considered for the manufacture of a live, attenuated influenza virus vaccine (VRBPAC 2008).

Cellular DNA emerged as a major safety issue. Thus, all possible functional DNA would need to be eliminated from the biological product with DNAses treatment and purification steps during the manufacturing process.

The current repertoire of cell substrates is inadequate for manufacture of certain types of new vaccines. To address this issue, the VRBPAC (2) recognized in 2012, that human tumor-derived cell lines could be an important addition to the repertoire of cell substrates for the manufacture of viral vaccines, and there was nothing that a priori precluded the use of such cells. This position was not confirmed by the European Medicines Agency (EMA).

In any case, the use of a specific tumorigenic cell substrate is generally discussed with the vaccines advisory committee before initiating a clinical trial. In particular, three major safety concerns need to be addressed: 1) the presence of residual live cells in the vaccine that might have the potential of being tumorigenic in humans; 2) the presence of residual DNA from the cell substrate; and 3) the potential presence of adventitious agents, including adventitious viruses that might have contributed to the tumorigenic phenotype.

Insect and avian cells.

The first continuously growing insect cell cultures were established from lepidoptera insects around 1960. Since then, the *Spodoptera frugiperda* Sf9 and *Trichoplusia* ni Hi-5 cell lines are the most widely used. The latter two are susceptible to the baculovirus *Autographa californica multiple capsid nucleopolyhedrovirus* and are used for the expression of foreign genes, for example, for the production of subunit vaccines to produce proteins for gene delivery vectors for mammalian cells. The baculovirus-insect cell expression system, often referred to as BEVS, is a well known tool for producing complex proteins and virus-like particles (VLPs) antigens providing rapid access to biologically active proteins.

The first commercially available veterinary vaccine produced in insect cells was the swine fever virus (CSFV) vaccine, and for humans medicine the European

Medicines Agency licensed in 2007 the bivalent human papilloma virus vaccine indicated for the prevention of cervical cancers.

Regarding avian cells, a stable duck cell line EB66 has been established from embryonic stems (ES) cells by spontaneous immortalization. After stabilization in a dedicated culture media, these cells were found to have an ultra structure similar to other ES cells, a large nucleus, expression of ES-specific markers on their cell surface, a stable karyotype, and to express the expected telomerase activity for a cell line derived from ES cells. A second category of duck cell line is the AGE1.CR1 which has been immortalized using E1 gene of adenovirus type 5, and which is grown in suspension cultures in serum-free media.

Stemcells

Stem Cells (SCs) differ from other cell types by sustaining a predominant stem-cell population that retains the capacity to produce cell progenitors of differentiated cell types of almost all human tissues.

Pluripotent SCs are well characterized and can be differentiated in different tissue specific cells showing some very interesting properties for virus productivity.

As for differentiated cell lines, the non transformed nature must be considered as a pre-requisite for vaccine production.

Unit V
Gene therapy

Mapping of human genome:

The genome of all living organisms consists of DNA, a very long two-stranded chemical polymer. Each DNA strand is composed of four different units, called nucleotides, that are linked end to end to form a long chain. These four nucleotides are symbolized as A, G, C, and T, which stand for the four bases—adenine, guanine, cytosine, and thymine—that are parts of the nucleotides. One DNA molecule, which together with some associated proteins constitutes a chromosome, differs from another in its length and in the order of its nucleotides. Each DNA molecule contains many genes, which are its functional units.

These genes are arranged in a defined order along the DNA molecule. Most genes code for protein molecules—enzymes or structural elements—that determine the characteristics of a cell. In bacteria, the coding sequences of a gene—are continuous strings of nucleotides, but in mammals the coding segments in a gene (called exons) are generally separated from one another by noncoding segments (called introns). Often each exon will encode a different structural region (or domain) of a larger protein molecule. Many exons have been found to be part of a family of related coding sequences that are used in the construction of many different genes (Doolittle *et al.*, 1986). Because of the many introns in mammalian genes, a single gene is often more than 10,000 nucleotides long, and genes that span 100,000 nucleotides are not uncommon.

For the information in the coding sequences of a gene to be expressed, the DNA of a gene must first be transcribed into an RNA molecule. Before the RNA strand leaves the cell's nucleus, the intron sequences are cut out of this RNA strand by a process called RNA splicing, thereby bringing the exon sequences into contiguity. Then the RNA can be translated into a protein molecule according to the genetic code (every group of three nucleotides codes

for one amino acid). Nucleotide sequences adjacent to the coding sequences in each gene encode regulatory signals for activating or inactivating transcription of the gene. Gene activity is a dynamic process; at any given time and in any given cell type, only a subset of genes is active. These active genes determine the course of embryological development and the characteristics of cells and organisms.

The Human Genome Is Composed of 24 Different Types of DNA Molecules

Human DNA is packaged into physically separate units called chromosomes. Humans are diploid organisms, containing two sets of genetic information, one set inherited from the mother and one from the father. Thus, each somatic cell has 22 pairs of chromosomes called autosomes (one member of each pair from each parent) and two sex chromosomes (an X and a Y chromosome in males and two X chromosomes in females). Each chromosome contains a single very long, linear DNA molecule. In the smallest human chromosomes this DNA molecule is composed of about 50 million nucleotide pairs; the largest chromosomes contain some 250 million nucleotide pairs.

The diploid human genome is thus composed of 46 DNA molecules of 24 distinct types. Because human chromosomes exist in pairs that are almost identical, only 3 billion nucleotide pairs (the haploid genome) need to be sequenced to gain complete information concerning a representative human genome. The human genome is thus said to contain 3 billion nucleotide pairs, even though most human cells contain 6 billion nucleotide pairs.

DNA is a double helix: Each nucleotide on a strand of DNA has a complementary nucleotide on the other strand. The information on one DNA strand is therefore redundant to that on the other (that is because of complementary base pairing, one can in principle determine the nucleotide sequence of one strand from the other). However, it is currently necessary to determine the sequences of the nucleotides on the two DNA strands separately to achieve the desired accuracy of any DNA sequence, with the sequence of

each strand being used as a check on the other. For this reason, a total of 6 billion nucleotides must actually be sequenced to order the 3 billion nucleotide pairs in the haploid human genome.

The average size of a protein molecule allows one to predict that there are approximately 1,000 nucleotide pairs of coding sequence per gene. Since humans are thought to have about 100,000 genes, a total of about 100 million nucleotide pairs of coding DNA must be present in the human genome. That this is only about 3 percent of the total size of the genome leads one to conclude that less than 5 percent of the human genome codes for proteins. The vast bulk of human DNA lies between genes and in the introns. Some of the noncoding DNA plays a role in regulating gene activity, while other portions are believed to be important for organizing the DNA into chromosomes and for chromosome replication (Alberts *et al.*, 1983; Lewin, 1987). The function of most noncoding regions of the human genome, however, is unknown; much of this DNA may have no function at all.

The Human Genome Can Be Mapped in Many Different Ways

It would be enormously useful to determine the order and spacing of all the genes that make up the genome. Such information is said to constitute a gene or genome map. Since there are 24 different DNA molecules in the human genome, a complete human gene map consists of 24 maps, each in the linear form of the DNA molecule itself.

One type of useful genome map is the messenger RNA (mRNA) or exon map. Cellular enzymes transcribe or copy all of an organism's genes into mRNAs so that the functions of the genes can be expressed. Complementary DNA (cDNA) of all the mRNAs present in an organism can be synthesized enzymatically with reverse transcriptase. These cDNAs can then be cloned and used to locate the corresponding genes on a chromosome map. In this way, the genes can be mapped in the absence of knowledge of their function. Another type of genome map would consist of an ordered set of the overlapping DNA

clones that constitute an entire chromosome. Both the exon map and the ordered set of DNA clones are usually referred to as physical maps. Alternatively, the position of a gene can be mapped by following the effect of the expression of the gene on the cells containing it. Here, a map is constructed on the basis of the frequencies of co-inheritance of two or more genetic markers. This type of map is referred to as a genetic linkage map

Maps of the human genome can be made at many different scales, or levels of resolution. Low-resolution physical maps have been derived from the distinctive patterns of bands that are observed along each chromosome by light microscopy of stained chromosomes. Genes have been physically associated with particular bands or clusters of bands in a number of ways. These associations permit genes to be mapped only approximately since a given gene might be assigned to a region of about 10 million nucleotides containing several hundred genes. Its exact position on the chromosome must be determined by more precise methods.

Maps of higher resolution are based on sites in the DNA cut by special proteins called restriction enzymes. Each enzyme recognizes a specific short sequence of four to eight nucleotides (a restriction site) and cuts the DNA chain at one point within the sequence (Watson *et al.*, 1983). Since dozens of different sequences are recognized by one or another enzyme, and these sequences are closely spaced throughout the genome, high-resolution physical maps can be constructed by determining the relative location of different restriction sites precisely. Of particular value in human gene mapping are restriction sites that are highly variable (or polymorphic) in the population. DNA lacking a specific restriction site yields a larger restriction fragment when cut by the enzyme than DNA containing the site; hence, the designation restriction fragment length polymorphism (RFLP).

Hundreds of polymorphic restriction sites have so far been identified and mapped in the human genome. Some disease-related genes have already been

localized by determining the frequency of co-inheritance of RFLPs and genetic diseases (Gusella *et al.*, 1983). Examples of these diseases include cystic fibrosis, Duchenne muscular dystrophy, Alzheimer's disease, and neurofibromatosis. Identifying a much larger number of useful polymorphic restriction sites should make it possible to map disease-related genes precisely enough to greatly facilitate the isolation of any human gene.

The map based on a collection of ordered clones of genomic fragments has a special value. In such a map, not only are the genomic positions of restriction fragments known, but each fragment is available as a clone that can be propagated and distributed to interested researchers. Such clones are immensely valuable because they serve as the starting point for gene isolation, for functional analyses, and for the determination of nucleotide sequences.

Only such a sequence reveals all or nearly all the information in the human genome. A number of specific regions of human DNA have already been analyzed in this way, providing information about the structure of genes and their encoded proteins in both normal and abnormal individuals and about sequences that regulate gene expression. At present, however, the nucleotide sequence of substantially less than 0.1 percent of the human genome is known. This includes the sequence containing 0.5 percent of our genes.

RFLP and Applications:

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. Polymorphisms are inherited differences found among the individuals in more than 1% of normal population.

Example of use of RFLP in DNA Typing:

1**. Extraction**: The first step in DNA typing is extraction of the DNA from the sample, be it blood, saliva, semen or some other biological sample

2. **Production of Restriction Fragments**: The purified DNA is then cut into fragments by RESTRICTION ENZYMES. Take the pattern GCGC and imagine it occurs more than once in the DNA. The number of times it occurs is unique to the individual. The restriction enzyme chops the DNA in two at every place where the GCGC pattern occurs. The restriction enzyme will cut between the first G and the first C.

3. **Electrophoresis**: The restriction fragments have negative charge and can be separated by a technique called GEL ELECTROPHORESIS, which separates the pieces of DNA based on their size. The samples of DNA that have been treated with restriction enzymes are placed in separate lanes on a slab of electrophoretic gel across which is placed an electric field. The fragments migrate towards the positive electrode, the smaller fragments moving faster than the larger fragments, thus separating the DNA samples into distinct bands.

4. **Detection:** The bands can be visualized using luminescent dyes. This approach to DNA typing required quite large samples of biological material in order to obtain reasonable results. For modern forensic work RFLP typing has been superseded by methodology based on the polymerase chain reaction which requires only minute amounts of sample for a successful typing.

Applications of RFLP:

RFLPs can be used in many different settings to accomplish different objectives.

1- RFLPs can be used in paternity cases or criminal cases to determine the source of a DNA sample. (i.e. it has forensic applications).

2- RFLPs can be used determine the disease status of an individual. (e.g. it can be used in the detection of mutations particularly known mutations)
3- RFLPs can be used to measure recombination rates which can lead to a genetic map with the distance between RFLP loci measured in centiMorgans.

DNA Fingerprinting:

The technique was developed in 1984 by British geneticist Alec Jeffreys.

DNA is the hereditary material presents in all the cells of the body. This molecule presents some characterizes, as VNTR, unique present in different individual. This is a key in the development of some techniques, in this case DNA fingerprinting. This procedure has simple steps that we will review in this work. DNA fingerprinting technique has become an important tool for scientific research, we will review some applications in fields like forensic investigations and parentage testing, moreover how this technique has revolutionized and evolved in areas as Anthropological genetics, botany and zoology.

What is DNA fingerprinting? In simple words, DNA Fingerprinting is the technology which is used to identify individuals on the basis of the molecular characteristics of the DNA. More specific, this method uses VNRT because the number of bases and repeats within a locus is unique to each individual. For example, an individual can have in his genome the sequence gatagata and this repeats 10 times and another can have the same sequence but only repeats 5 times. The technique is used, as we have seen before, in parentage testing and forensic cases but it can be used for anthropological genetics, zoology, and botany among others disciplines. Importantly, the technique of DNA Fingerprinting is very sensitive, which means that it can also generate data even from half (partially) decomposed biological material. Procedure to create a DNA fingerprinting. The steps involve others techniques used in Molecular Biology, such as polymerase chain reaction (PCR) and

electrophoresis among others. The following are the steps to generate a DNA fingerprinting.

1. The DNA is extracted from the nuclei of any cell in the body.

2. The DNA molecules are broken with the help of enzyme restriction endonuclease (called chemical knife) that cuts them into fragments. The fragments of DNA also contain the VNTRs.

3. The fragments are separated according to size by gel electrophoresis in agarose gel.

4. The separated fragments of single-stranded DNA are transferred onto a nylon membrane. Radioactive DNA probes having repeated base sequences complementary to possible VNTRs are poured over the nylon membrane. Some of them will bind to the of single-stranded VNTRs. The method of hybridization of DNA with probes is called Southern Blotting.

5. The nylon membrane is washed to remove extra probes.

6. An X-ray film is exposed to the nylon membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is developed. This is known as autoradiography.

7. The dark bands on X-ray film represent the DNA fingerprints (DNA profiles) .

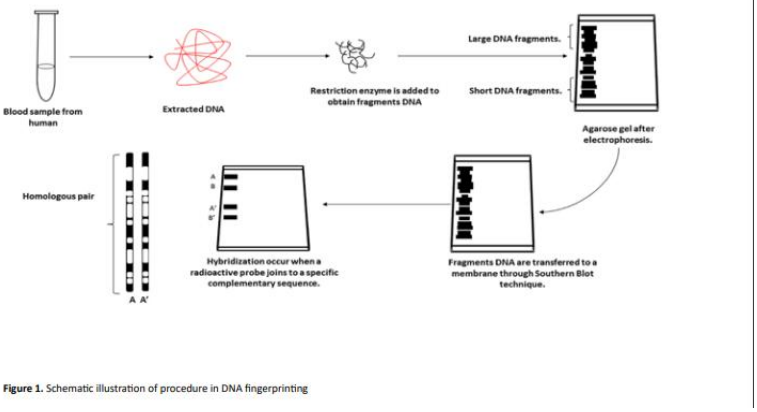


Figure 1. Schematic illustration of procedure in DNA fingerprinting

Forensic Science – Chemistry, Physics, Biology, and Engineering:

The word "forensic" originates from the Latin word forensis, which means public, to the forum, or public discussion. A modern definition of "forensic" is relating to, used in, or suitable to a court of law. Any science used for the purposes of the law is a forensic science. Forensic sciences deal with the application of scientific knowledge to legal problems and they are vital tools for unearthing the truth in any legal proceeding. Forensic sciences, including forensic chemistry, forensic biology, forensic anthropology, forensic medicine, forensic materials science, forensic engineering, computational forensics, and so on, are broadly used to resolve civil disputes, to justly enforce criminal laws and government regulations, and to protect public health. In some subareas of forensic science, for example, in forensic botany, simple observation of plant samples collected at the crime scene could be enough for arriving at important conclusions, while in other forensic methods, such as forensic chemistry, sophisticated instrumental analytical methods are required.

The most frequently encountered examples of forensic science applications are fingerprints and DNA analysis, both aiming at the identification of crime victims or criminals. However, forensic science methods go much beyond these well-known applications and often include various physical and chemical analytical methods. Vibrational spectroscopy (based on IR absorption and Raman scattering), internal reflection spectroscopy, mass

spectrometry, and electrochemistry have been applied for forensic analyses of human or animal hair, fiber, paints and inks, and a variety of human body fluids, as well as for the detection of gunshot residues, controlled substances (e.g., illicit drugs), explosives, and other chemical and biological agents. Spectral analysis of objects found at the crime scene can be subjected to hyperspectral imaging (HSI) to obtain both spatial and spectral information from the sample. This technique enables investigators to analyze the chemical composition of traces and simultaneously visualize their spatial distribution. HSI offers significant potential for the detection, visualization, identification, and age estimation of forensic traces, also allowing forensic analysis of document forgery.

A biochemistry-/molecular biology-based subarea of forensic analysis, called forensic serology, deals with the complex task of gathering information on the type Forensic Science. Forensic electrochemistry – the electroanalytical sensing of gunshot residues of sample, age, origin, or sex from biological fluids (blood, saliva, etc.) found at a crime scene. Analysis of various biomarkers in biofluids found on the crime spot can help in arriving at preliminary conclusions about the race, sex, age, and so on, of possible suspects. DNA typing of criminal suspects or victims can be extended to the DNA analysis of human remains as well as to the analysis of DNA damage and repair in forensic samples. Forensic analysis of blood stain patterns has become one of the most frequently used and highly important procedures providing key evidence with its ability to potentially map the sequence of events, highlight movement through the crime scene, and identify the minimum number of blows executed.

Other materials, particularly left at trace levels, are attracting the attention of forensic investigators, being highly important for the reconstruction of the performed crime. Personal identification methods, for example, based on fingerprints, as well as possible complications originating from their spoofing represent an important part of the forensic study. Various engineering disciplines, including mechanical, electrical, and chemical

engineering, fire science, and so on, are also involved in forensic investigations, often in civil cases but also in criminal investigations.

Molecular diagnosis of genetic disorders:

The use of molecular biology techniques to expand scientific knowledge of the natural history of diseases, identify people who are at risk for acquiring specific diseases and diagnose human disease at the molecular level.

- Analysis of human DNA, RNA, Chromosomes, proteins and metabolites.
- To detect heritable disease related to genotype, phenotype and karyotype.
- For clinical purpose.

Polymerase chain reaction:

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis.

The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids.

It's a means of selectively amplifying a particular segment of DNA. The segment may represent a small part of a large and complex mixture of DNAs e.g. a specific exon of a human gene. It can be thought of as a molecular photocopier. PCR can amplify a usable amount of DNA (visible by gel electrophoresis) in ~2 hours. The template DNA need not be highly purified — a boiled bacterial colony. The PCR product can be digested with restriction enzymes, sequenced or cloned. PCR can amplify a single DNA molecule, e.g. from a single sperm. The polymerase chain reaction relies on the ability of DNA copying enzymes to remain stable at high temperatures. PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a result of its simplicity and usefulness.

In Mullis's original PCR process, the enzyme was used in vitro. The double-stranded DNA was separated into two single strands of DNA by heating it to 96°C. At this temperature, however, the E.Coli DNA polymerase was destroyed, so that the enzyme had to be replenished with new fresh enzyme after the heating stage of each cycle. Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process.

Steps in PCR There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complementary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle. To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates.

This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on. The annealing phase happens at a lower temperature, 50-60°C. This allows the primers to hybridize to their respective complementary template strands, a very useful tool to forensic chemistry. The newly-formed DNA strand of primer attached to template is then used to create identical copies off the original template strands desired. Taq polymerase adds available nucleotides to the end of the annealed primers. The extension of the primers by Taq polymerase occurs at approx 72°C for 2-5 minutes.

DNA polymerase I cannot be used to elongate the primers as one would expect because it is not stable at the high temperatures required for PCR. The beauty of the PCR cycle and process is that it is very fast compared to other techniques and each cycle doubles the number of copies of the desired DNA strand. After 25-30 cycles, whoever is performing the PCR process on a sample of DNA will have plenty of copies of the original DNA sample. Assuming the maximum amount of time for each step, 30 cycles would only take 6 hours to complete. As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2n, where n, is the number of cycles. Due to the introduction of a thermostable DNA polymerase, the Taq DNA polymerase once, at the beginning of the PCR reaction. The thermostable properties of the DNA polymerase activity were isolated from Thermus aquaticus (Taq) that grow in geysers of over 110°C, and have contributed greatly to the yield, specificity, automation, and utility of the polymerase chain reaction.

The Taq enzyme can withstand repeated heating to 94°C and so each time the mixture is cooled to allow the oligonucleotide primers to bind the catalyst for the extension is already present.10 After the last cycle, samples are usually incubated at 72°C for 5 minutes to fill in the protruding ends of newly synthesized PCR products. To ensure success, care should be taken both in preparing the reaction mixture and setting up the cycling conditions. Increasing the cycle number above ~35 has little positive effect because the plateau occurs when the reagents are depleted; accumulate. The specificity of amplification depends on the extent to which the primers can recognize and bind to sequences other than the intended target DNA sequences.

Transgenic:

Merits- Transgenic Animals

1. This science allows us to create specific economic traits through animal products.

Some transgenic animals are produced for their specific economic traits. Cattle can produce milk that contains particular proteins which could help in the treatment of emphysema. We use antibodies from mice and other rodents to create new medical therapies that can reduce or eliminate the impact of illness and disease. Harvard scientists received a U.S. patent for a genetically-engineered mouse that carries a gene which promotes the development of human cancer so it can be studied more effectively.

2. There are three different methods available to use to create transgenic animals.

Researchers can take advantage of three common methods of producing transgenic animals for their research. Genes can be transferred to the animal through DNA micro-injection, by using a retrovirus-mediated transfer process, or to do so through embryonic stem cell transfers. Most facilities still use the first method, and then the transgenic animals are mated to ensure that their

offspring acquire the desired genetic profile. This advantage allows for the traits to become part of the animals' standard genome over just a generation or two.

3. It allows us to research human diseases more effectively.

Transgenic animals make it possible for scientists to study the functioning of particular genes at the level of the entire organism. This process enhances the study of physiology in a number of fields in addition to the benefits achieve in disease biology. New targeted drugs have come from this work, but the similarity in function and physiology between humans and rodents makes it easier for the laboratory to use this technology and process to mimic human disease.

Transgenic mice are currently being used as models to study heart disease, arthritis, anxiety, Alzheimer's disease, diabetes, aging, and Parkinson's disease. We can also glean new insights into how cancer forms with this process.

4. We could potentially solve our organ transplant list crisis.

There are currently more than 114,000 people on the waiting list for a life-saving organ transplant in the United States right now. Thousands more are on lists all over the world. Another name is added to it in the U.S. lists every 10 minutes on average. About 20 people die each day because there is a lack of organ availability. Transgenic pigs could help to solve this problem. Researchers are looking at these animals as a potential source for organs to transplant, which would allow us to reduce severe donor shortages.

5. This technology can help us to create life-saving medicines already. We are already using transgenic technologies as a way to create insulin and other maintenance medications that are challenging to synthesize otherwise. Animals who have their genes manipulated can help to produce more of the vaccines that are necessary to reduce the threat of serious communicable diseases. Blood clotting factors are possible with this advantage as well.

Transgeneis makes it possible to save lives proactively because we are using natural methods of product creation.

6. It gives us a way to create healthy additives for our food supply. Lactoferrin is a protein that is found in human breastmilk and bovine milk. The colostrum that comes immediately after a baby is born contains significant amounts of it. You can also find it in the fluids of the intestine, nose, respiratory tract, and even in your eye. When it is used as a medicine, it can treat stomach ulcers, intestinal problems, diarrhea, and even hepatitis C. It functions as an antioxidant too, which means it can help to protect against viral and bacterial infections. It even stimulates the immune system.

Transgenic cattle who can produce milk with higher quantities of lactoferrin can create robust supplies that are useful in numerous industries. Even industrial agriculturalists use it to kill bacteria during their meat processing work.

7. Transgenesis can produce animals which are stronger and healthier. Although the success rate for artificial breeding is low when looking at the transgenic technologies today, scientists can naturally breed the animals that come from their work. When both parents have the same manipulated traits, then the odds of having it pass along to the next generation are exceptionally high. We can begin to build a healthy and helpful generation of animals from a variety of species that are productive, useful, and healthy without being abusive to them.

8. It can reduce the environmental impact of the animals.

Commercial farming over the past generation has seen numerous criticisms over the number of adverse environmental impacts that occur from their processes. The production of transgenic livestock brings with it the potential to dramatically reduce the footprint of this agricultural economic force without limiting its value. Transgenesis could increase efficiencies while

reducing the amount of water and land resources needed to support large herds. It could even work to protect the groundwater and soil.

Pigs do not utilize phosphorus well, so they require continuous supplements to ensure proper health. Not only does this increase production costs, but it can also change the composition of the soil. Transgenesis could change the genes which turn off absorption for the swine so that farmers see lower costs, higher yields, and improved land resources simultaneously.

9. Transgenic animals can improve the quality of our food supply. We might use transgenic animals as a way to improve access to medicines and research, but this technology is also useful in the creation of better food products. Enhanced milk is a common option considered with this benefit. The three primary nutrients that are in this fluid are lactose, fat, and protein. By elevating or decreasing them, we can impact the health and growth of animal offspring while improving the items on store shelves as well. All it takes is an improvement in milk yields of as little as 2L per day to have an immediate impact on the weaning weight of cattle.

10. This technology improves disease resistance for the animals too. Genetic modification of livestock can enhance animal welfare because it creates the potential of having a healthier animal. The agriculture industry places the welfare of livestock as a high priority because the loss of single individual can mean several thousands in lost profits. The application of transgenesis methodologies will provide opportunities to create a superior level of disease resistance. Whether there is a need to treat mastitis, manage hoof rot, or improve the overall performance of the animal, this technology can make good things happen.

11. Transgenesis can improve the quality of non-food animal products. The quality, yield, and color of the fiber, wool, and hair that we use that gets harvested from animals has long been a focus of the transgenic animal industry. By manipulating the length, strength, and crimp of the wool, there

could be an increase in the availability of fibers without impacting the overall cost to the consumer. By decreasing the surface interaction of each fiber, it may become possible to decrease shrinkage in the apparel made from these fibers as well.

12. We can use this technology to create better agricultural products from other animals.

One of the most novel approaches that scientists have created through transgenic animals is an improvement to the production of spider silk. This useful fiber uses the milk of transgenic goats. Instead of waiting for orb-producing spiders to weave a web, the technology allows for the synthesizing of up to 7 different types of silk. The Dragline variety is the most coveted as it can be elongated by up to 35%, offers robust tensile properties that are equitable to Kevalr™, and it offers an energy-absorbing capability that is stronger than even steel.

Demerits- Transgenic Animals

1. There are numerous ethical concerns to review when using transgenic animals.

We must take a look at the decision-making processes from an ethical standpoint to determine if the creation of transgenic animals is the right choice to make. Although some people will take an "ends justifies the means" approach to this situation, we must at least look at the various processes involved to determine if there should be a universal protocol that researchers follow. Then we must ask if human welfare is the only consideration in the evaluation process. Do we need to focus on cultured methods instead of using live animals?

2. It creates the potential for economic harm due to the patenting process.

The creation of the OncoMouse® was a significant breakthrough for research science, but it also set a dangerous precedent from a patenting standpoint.

When companies can protect their research in this manner while manipulating the genome of animals, then the motivation becomes more about the potential for profits than it does for human welfare. That makes it a challenge to present an ethical defense because you're exploiting live animals to create a healthier profit margin.

We butcher animals all of the time as a food resource, but the argument that killing an animal is no better than harming one is a false equivalence. Using live creatures to exploit others sets the stage where only the wealthy can gain access to this new technology.

3. The reliability of transgenic technologies is questionable.

The success rates of creating transgenic animals using DNA micro-injection are extremely low, even though this method is the most popular one used by researchers today. Only 0.6% of transgenic pigs were born with a desired gene after workers injected over 7,000 eggs with a specific transgene. That means we are introducing a lot of waste into the system where the animals may not even have a chance to live.

4. There is no consistency to the patenting process in this industry.

The Supreme Court of Canada ruled in 2002 that higher life forms are not eligible for patenting because they were not a composition or manufacture of matter within the meaning of an invention in accordance to the Patent Act. The United States issued a patent for the OncoMouse without question, which is No. 4,736,866, although it is notable that the claim explicitly excludes humans. Europe approved this patent eventually because the usefulness of cancer research satisfied the likelihood of a substantial medical benefit, but rejected the Upjohn mouse because the gene only caused the animals to lose their hair.

In the 1992 Upjohn case, the pharmaceutical company wanted to test products that could increase wool production and treat human baldness. The European Patent Office decided in this situation that the harm to the animals outweighed the benefits that society would receive from the work. The reality is that there

is no consistency across nations as to how we should commercially treat transgenic animals.

5. It might be dangerous to eat transgenic animals.

One of the advantages that transgenic technology supporters often point out is that this work can create healthier animals who can provide us with a better nutritional profile. Americans and Europeans are sharply divided on this subject. The application of gene modification technologies is strictly regulated for domestic and imported goods. Providers must follow the legal frameworks in place before they can even start the process of working with transgenic animals. No GM animals or derived products are on the market in Europe, but they could be in the United States.

No documented cases of harm have been documented from a clinical standpoint to justify the concerns of allergies or illness from genetically modified foods. We also have no data on how the long-term health of individuals could be affected by consuming these items regularly.

6. Transgensis could result in the unregulated expression of genes.

Although scientists feel like they have a handle on the processes necessary to create transgenic animals and breed new generations of them, there is always the risk that this technology could create unregulated expressions that could over- or under-produce the wanted products from this effort. Having too much of the wanted item might seem to be a nice problem to have, but it can also impact the lifespan of the animal.

Cows have a natural lifespan of approximately 20 years. Some species can produce milk for eight or nine years. The stresses of constantly producing a food supply, coupled with factors that include lameness or disease, can shorten that time by up to 50%. It is not unusual for the dairy industry to send cows for slaughter (even transgenic animals) before they reach the age of 5.

7. It comes with the possibility of side effects for some of the animals.
There are numerous potential side effects that transgenic animals might experience after they receive gene manipulation treatments. Even if this process occurs in the womb, evidence from transgenic swine showed that the animals were more prone to developing arthritis, had changes to their skeletal growth, suffered from dermatitis and gastric ulcers, along with an increase in renal disease and cardiomegaly. When these problems occur in the genetic profile of the animal, then it becomes a waste of resources for everyone involved in the development process.

8. Transgenesis can result in a higher level of genetic mutations.
One of the most significant risks of transgenesis technologies is the risk of an insertional mutation occurring. This problem occurs when a DNA fragment goes into an essential gene for the animal. It can result in numerous biological processes being altered in ways that are not beneficial to the animal or the work being done. Mosaicism is also possible because of this technology, since there is always the possibility that only a small portion of the cells will actually incorporate into the transferred gene.

Researchers have also discovered that there are times when the transgene integration process only occurs on the Y chromosome. When this issue happens, then the desired traits will only transfer through the males of the species. Most of the issues are related to the actual transgene, the integration site, or its expression and can be addressed, but there are still unpredictable factors to review as well.

Ethical issues in animal biotechnology:

Animal Biotechnology: Ethical Challenges Despite potential outcome from animal biotechnology, there are a number of controversies regarding several areas of the application of animal biotechnology. If we concentrate only on the benefits of human beings in terms of the consequences, possibly we will not find any problem in it. There are also dark side of biotechnology (as well as of

animal biotechnology), which cannot flight away our insight. A number of ethical experts have realized the adverse effect of this technology. Gerhald Becker compares this drastic implication and the social impact of this technology with "the splitting of the atom and the technological exploitation of nuclear power. As with nuclear technology, biotechnology has put enormous power in our hands" (Becker, 1996: 4). Nuclear power can contribute positively to the well-being of mankind. At the same time, it can destroy innumerable lives of human beings. In the similar way, biotechnology has also got same evil purposes, which would cause "incalculable risks for human integrity, well-being and freedom" (Becker, 1996:5). Some other experts like J. Thomson (1996) argue that these risks can be transformed into moral concerns. He focuses on the 'unintended consequences' and 'ethical concerns' of modern biotechnology, which is "inherently unethical" (Thomson, 1996:123-124).

The development in biotechnology during the last few decades has raised a lot of ethical controversy. Critics have generated different arguments while opposing this technology, which may conveniently be divided into two kinds: (1) intrinsic arguments and (2) extrinsic arguments (Kaiser, 2005:75). Intrinsic argument against biotechnology maintains that biotechnology is "objectionable in itself" (Comstock, 2000: 76). And extrinsic argument focuses on the "allegedly harmful consequences of making GMOs" (Comstock, 2000: 76). In this sense, animal biotechnology is ethically problematic because "it is unnatural to genetically engineer plants, animals and foods" (Comstock, 2002:76). The argument goes like this, biotechnology is the form of 'redesigning an animal' which is the "Playing with God". (Animals) biotechnologies are also break down the natural species boundaries. In the sense of extrinsic argument, animal biotechnology is ethically wrong because of its negative consequences on human beings, animals, and environment. Intrinsic Arguments against Animal Biotechnology

i. The argument of Playing with God is based upon the concept of 'God's will' and on the relationship among God, nature, animals, and human

beings. He thinks that the view that God himself sets out a plan and makes designs for the universe and human beings is being assigned to observe it. God as an omnipotent and omniscient being, has set out a specific 'roadmap' for the universe, animal kingdom, and nature (Coady, 2009:155-180). But, animal biotechnology tempers the animals' design by inserting a new gene into a species. Thus, in a way (animal) biotechnology breaks down the boundary between the 'realm of God' and the 'realm of humans'. Is the 'playing with God' argument enough to oppose animal biotechnology? We get responses to such a question in Ronald Dworkin's book Sovereign Virtue (2000) in which he argues that in the bio-political context 'the argument for Playing with God' is not 'morally and intellectually honest'. This is not a recent phenomenon to sustain the fight against the hostile nature. Human beings, for their necessity and needs, rearrange nature in the way they find it suitable for them. Biotechnology is such a technology that has essentially become a part of human life. Therefore, the argument for the Playing with God is not a strong stand to stop biotechnology.

i. Break-down of the Natural Species Boundaries Recently, a conceptual study, "Ethical Aspects of Agricultural Biotechnology" (BABAS, 1999) has shown that any sort of biotechnology is morally unacceptable because of its 'unnaturalness' (AEBC, 2002). The European Commission agrees with the idea that (animal) biotechnology is 'unnatural'. This theory also indicates that the application of biotechnology breaks the natural order of different kinds of species. Something natural is assumed to be valuable and good. But, all kinds of biotechnology or genetic technology temper nature where species boundaries are crossed. The term, 'Natural', is somehow different from the concept 'Unnatural'. The difference can be shown as follows: "Nature and all that is natural is valuable and good in itself; all forms of biotechnology are unnatural in that they go against and interfere with Nature, particularly in the crossing of natural species boundaries; all forms of modern biotechnology are therefore intrinsically

wrong" (BABAS, 1999 : 10). Something, which is natural also, means that it is 'normal', 'right', 'appropriate', and 'suitable'. On the contrary, 'unnaturalness' refers to something which is man-made, artificial, 10 or which is dependent upon our interference with the natural world. 'Unnaturalness' has got a broad spectrum in our modern life. For example, most of the food production, animal farming, clothing, and used materials are the result of unnatural interference of nature. 'Naturalness' and 'unnaturalness' can be characterized as 'non-anthropocentric view' and 'anthropocentric', respectively. The anthropocentric view proposes a careful management of resources along with interference of nature. On the other hand, the eco-centric view holds non-interference in relation with nature. The eco-centric view accompanies the view of 'respect for nature', which does not allow any biotechnological tool as a means of the interference of nature. As an anthropocentric means, biotechnology is the viable example of 'unnaturalness' by which natural integrity of species and the species boundaries are breached.

ii. Animal Integrity and Animal Biotechnology Does animal biotechnology violate the concepts of 'animal integrity'? The Dutch National Committee on Animal Biotechnology presents their argument that biotechnology has got potential negative effects upon animals. For the sake of human benefits, we changed the properties of animals by genetic modification, which is the 'violation of the integrity of the animal'. Before entering into the objection against the application of animal biotechnology, we will shortly clarify the concept of 'animal integrity'.

   a. The concept was not developed in the biotechnological context. Rather, it was borrowed from the field of ethics for making an assessment of the impact of animal biotechnology and the genetic modification of animals. In the Utrecht University, a number of ethicists and veterians give their definition of the term, 'animal integrity'. In the definition, they stress on the fact that every animal

has (i) 'wholeness and completeness', (ii) species-specific balance of an animal and (iii) animals have its own capacity to maintain itself independently in the environment suitable to the species (Vries, 2006: 471, Rutgers & Heeger 1999: 41-51, Heeger, 1997: 243-252). The notion about 'animal integrity' implies that we should not apply any sort of interference upon all these features of animals. The definition mentioned above implies that every animal has got its own 'physical intactness', which is not expected to be interfered. However, genetic engineering and trans-genesis process of animals introduce "a gene foreign to the species to a gamete, the wholeness and completeness of the animal [...] is altered at its most fundamental level, the genome" (Vries, 2006: 471). So, it is now understood that any form of animal biotechnology is rather a kind of interference in the 'wholeness and completeness' of the animal. For example, a chicken or a pig has got some characteristic features of its own, which should not be changed by tempering 11 its original physical intactness. But, breeding and transgenic process applied in broiler chicken and pigs certainly violate the wholeness and completeness of animals. Rutgers and Heegers have described the second feature of the animal integrity thus: 'species-specific capacities'. They assume that the violation of the second feature is the violation of principle of animal integrity. For example, after the production of a broiler chicken, it grows very fast. But, it cannot move naturally. It grows rather in a very abnormal pace and its biological fitness is not suitable for the environment. All such things upset its biological balance. Rutgers and Heeger claim that "the more the animals lose its species-specific capacities and characteristics, the more serious the integrity violation" (Rutgers and Heeger, 1999: 49). The third characteristic feature of animal integrity is environmentally suitable for the animal. The moral status of an animal is another prime issue regarding animal integrity. Different questions can be raised in this regard: what is the ethical status of

the species? Is there more ethical importance of some species than the others? Besides, there is also controversy amongst the thinkers as to whether animals have any moral status or not. Besides, in what sense should animals be considered morally? Heeger answers to this question thus: "animals have good of their own" and "they have interests, namely in everything that contributes to the realization of their good" (cf. Vries, 2006: 473). Biotechnology intervenes with the intact body of animals and also threatens their essential characteristics. So, it can be said that animal biotechnology does not maintain any good/ well-being of animals.

Extrinsic Argument- deals with two potential questions:

i. Does animal biotechnology violate the criteria of 'animal welfare'?
ii. What are the effects of biotechnological application upon the environment?

Animal Welfare- Before finding out the answer to the first question, at first, we shall have to make the concept of 'animal welfare' clear. The term, 'animal welfare', is used by different types of people, specially by veterinarians, farmers, consumers, and politicians. Veterinarians focus on the physical environment such as shelter and feeding; they also need to measure how the animals are coping with the existent environment (Brom, 1991: 4167-4175). Besides, there are people who think it is important to maintain the psychological status of animals. They are of the opinion that animals have various psychological states such as fear, frustration, and pain, which need to be addressed. It should be taken as part of their primary needs (Duncan, 2002: 643:652). So, it can now be said that the overall physiological and 12 mental well-being of the animals is called animal welfare. However, application of animal biotechnology affects animal welfare in the following two ways: (a) By using biotechnology, different kinds of animal drugs and feed additives are produced which have adverse effects on the animal health. In genetic rearrangement, either in the non-sexual or in the sexual exchange, or in the laboratory, unavoidable sufferings of animals is beyond description. A study

(BABAS, 1999: 22) shows that after breeding farm animals suffer from infections from Rota-Viruses, which are caused by heavy diarrheadiseases. These viruses damage their intestinal mucosa. The same study gives an example of the experiments conducted on the pigs through which they were genetically modified. By inserting additional gene copies for growth hormone into pigs, it is possible to bring forth faster growth of the offspring. But, the animals involved in the process suffer from severe arthritis, which affects their health seriously. (b) Application of animal biotechnology involves such procedures that can cause types of different sufferings for the animals.

There are different sorts of proceedings which are related to animal biotechnology. First of all, it encourages the use of a large number of animals within a limited place. Intensive livestock farming is one of them. What happens in this kind of farming is made clear by the statement given by Peter Singer. He opines that there is no tolerable life for the animals who are under in intensive livestock farming. There, throughout the year, animals are crowded in a battery cage, or in the cases of a breeding sow, there they are unable to walk or turn around, there is no way of socializing, sometimes they are thrown out and killed. All these steps are evidences of ill-treatment of animals as these confines them to a limited boundary (Singer, 1989, evidence: 9470). Animals are also deprived of their necessary ethological and biological needs. In this kind of farming, caging, restraining, spacing, breeding, roaring, slaughtering, controlled environmental situation are common phenomena. Do animals enjoy 'freedom' due to biotechnological application? Brambel has produced a seminal report on animal welfare, which refers to the existence of 'five freedoms' as the condition of animal welfare. These are : a). "freedom from hunger and thrust — by ready access to fresh water and a diet to maintain full health and vigour"; b). "freedom from discomfort — by providing an appropriate environment including shelter and comfortable resting "; c) "freedom from pain, injury or disease — by prevention or rapid diagnosis and treatment"; d) "freedom to express normal behaviour — by providing sufficient space, proper facilities and company of the animal's own kind"; e) "freedom from fear and distress — by

ensuring conditions and treatment that avoid mental suffering" (Brambel Report, 1965, Report : 2836, cf. Kaiser, 2005 :80). Some 13 genetically modified animals suffer from pain. They cannot behave normally due to their deplorable physical condition. Application of some specific kinds of biotechnology such as the use of bovine somatotrophin (bST) on animals results in the physical sufferings which seem to be an act of violation of said five freedoms.

iii. Environmental Concern. In response to the question (ii), scientists have put forward different kinds of arguments. A study on 'animal biotechnology and environment' accomplished by Krimsky and Wrubel (1996), claims that animal biotechnologies have got an enormous amount of environmental benefits. They argue that in the traditional milking system more cows give less amount of milk and occupy more agro-land, more cows also produce more slurry and manure. On the other hand, the use of biotechnology is helpful in reducing the amount of land required; thus it can keep the land for non-agricultural purposes. Another study has shown that a genetically modified animal generates 'low phosphorus manure' (Goloven, et.al. 2001: 741-745). Usually, feed phosphorus from animal manure is responsible for the pollution of surface water. Low phosphorus contributes TO less pollution. Thus, the use of biotechnology turns into a great environmental benefit. Interestingly, there are a number of studies which argue that the application of animal biotechnology causes a lot of environmental problems. Application of this technology in the food sector, particularly in milk, meat, and egg productions has made the demand for intensive livestock farming. Various studies have shown that livestock farming, including intensive livestock farming, is responsible for greenhouse gas emission. It also claims that intensive livestock farming (including livestock) is "probably the largest sectoral source of water pollution, contributing to eutrophication, "dead" zones in coastal areas, degradation of coral reefs…" (LEAD, 2006: 17). 2.3 An Evaluation Regarding the intrinsic

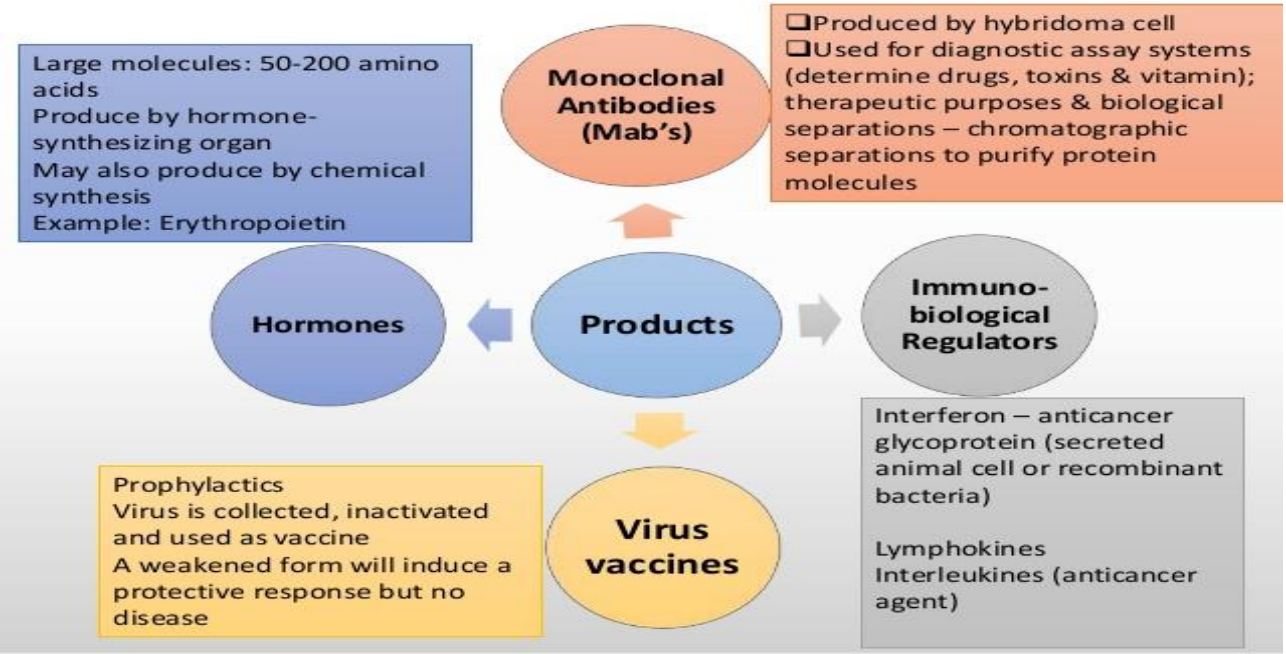
argument we can explore the following two points at least: Firstly, the central theme of intrinsic argument is that every species has got its own shape and structure, which it gains in a natural way. Natural diversity refers to the existence of particular characteristics of every species. Some animal biotechnologies such as transgenesis and Xenotransplantation break-down the natural diversity of animals, which is not right way of treating them. In response to this criticism, we can mention here theory of Darwin's theory of evolution. According to this theory, the structure and the phase of every species is not static. According to Darwin (1859), phenotypes of species change from one generation to the other over a long period. Various new types of species arose from the species of the past through a process of gradual change. The period of change might be as long as hundreds of years or even 14 more than that. Species are also changing their physiological structure, either by natural selection or by their adaptation to the environmental changes. Sometimes, the course of change in the animal occurs in its inner genetic mapping. Most of the theorists of evolution regard this change as a natural process. The natural change of animals might occur slowly over the years. There is another example we can explicate here. Some of the viruses have capacity to bear genetic materials which are very much helpful for gene transformation to another species. This gene can bring a radical change in the new species. This is a natural process of change as it occurs through biotechnological process. So, the idea that is not based on strong arguments as such a break-down of natural species has always been occurring in the animal kingdom. Secondly, sometimes animal biotechnology is considered as unnatural, which is intrinsically wrong. Do we think that in the natural world anything natural is normal or ethical? Regarding this question, we can refers to some of natural phenomena such as earthquake, cyclone, storm, drought, flood, and many other such natural calamities which usually take place in nature and create an abnormal phenomenon. Although it is described as

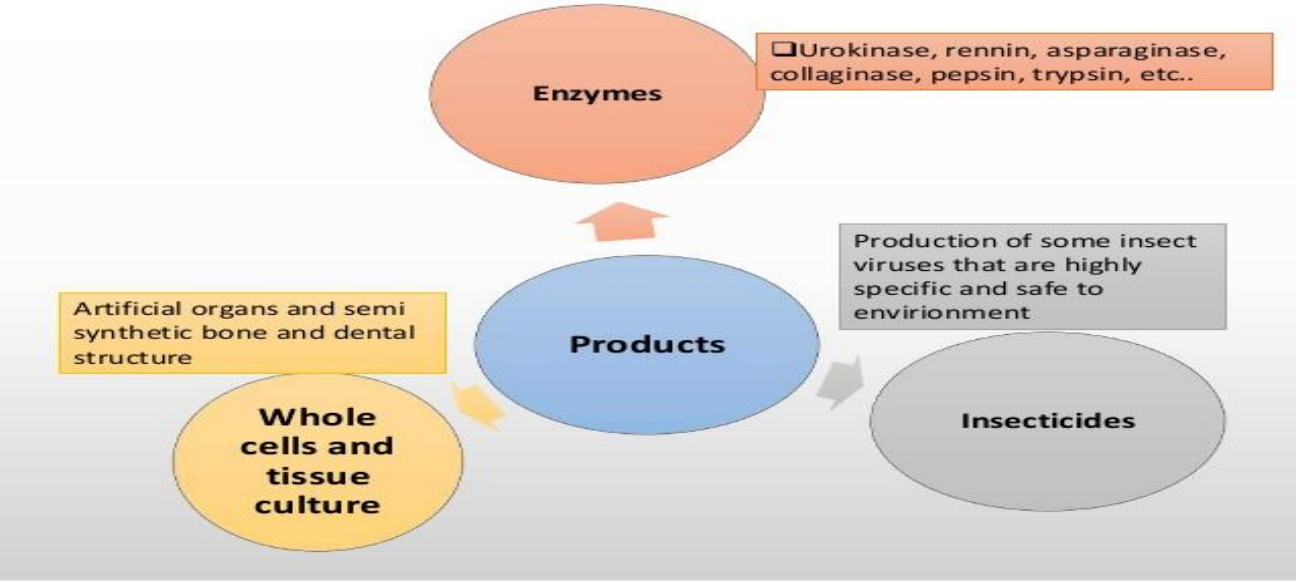
'natural' should we consider it as normal or intrinsically good? Of course, we do not consider these as normal phenomena. So, something that is natural or formulated by natural law does not always mean that it is arranged or created by the law of order or in a disciplined way. In this sense, the concept, 'natural' does not mean good or normal as it is attributed by the critics of animal biotechnology. If we look the agricultural crops and food by which we live, we can realize that these are the results of biotechnological formulation.

iv. The system of production of agricultural crops is the best instance of biotechnology. Even in the animal kingdom naturally and artificially there is a verity of the forms of biotechnology. We mould the nature for our suitable use by applying certain techniques upon it. So, the techniques for processing nature, the techniques for producing crops, and the techniques for creative survival and progress of dwelling are the essential features of our living. Regarding the concept of 'extrinsic argument', it has been argued that new technologies used in animals cause pain and sufferings in different ways. But, there are also opposite views to it. Animal biotechnology such as cloning or transgenic technique does not necessarily cause pain to an animal. Rather, it reduces the animal's pain. Furthermore, it can be said that in the conventional system of animal breeding an animal experiences severe pain (EGE, 2008:22). Not only that, the conventional style of domestication also violates 'animal integrity' and 'animal welfare'. For example, in the domestication system, animals are infringed in a limited 15 boundary; its movement is confined to that area, and its feeding and natural requirements are met and determined from the outside. However, to get a balanced life and physiological growth animals need suitable environment where they can grow naturally and smoothly. [Bio]Technology (whether it is animal or agricultural) is one of the means of our living today. We cannot deny or oppose it all on a sudden. We need to be careful as well as critical in this regard. Therefore, it is an

imperative that we select tools for better assessment for evaluating [bio] technology. In order to assess the ethical issues raised on the application of biotechnologies in agriculture and food production, a number of countries belonging to the European Union have developed a method of decision-support tools. A group of ethical experts has developed a set of ethical tools which is known as Ethical Bio-TA. It facilitates ethical decision-making by the government agencies, the general public, and the financial actors in the food chain.

Production and recovery of products from animal tissue:





Applications of Animal Cell Cultures

There is a widespread concern that extensive use of animals for laboratory experiments is not morally and ethically justifiable. Animal welfare group's world over are increasingly criticising the use of animals. Some research workers these days prefer to utilize animal cell cultures wherever possible for various studies. The major applications of laboratory animal cell cultures are given in Table 33.3, and listed below.

Table 33.3 Summary of the applications of animal cell cultures

Category	*Applications*
Intracellular activity	Studies related to cell cycle and differentiation, transcription, translation, energy metabolism, drug metabolism.
Intracellular flux	Studies involving hormonal receptors, metabolites, signal transduction, membrane trafficking.
Cell to cell interaction	Studies dealing with cell adhesion and motility, matrix interaction, morphogenesis, paracrine control, metabolic cooperation.
Environmental interaction	Studies related to drug actions, infections, cytotoxicity, mutagenesis, carcinogenesis.
Genetics	Studies dealing with genetic analysis, transfection, transformation, immortalization, senescence.
Cell products	Wide range of applications of the cellular products formed (Refer ***Table 33.4***) e.g. vaccines, hormones, interferons etc.

i. Studies on intracellular activity e.g. cell cycle and differentiation, metabolisms.

ii. Elucidation of intracellular flux e.g. hormonal receptors, signal transduction.

iii. Studies related to cell to cell interaction e.g. cell adhesion and motility, metabolic cooperation.

iv. Evolution of environmental interactions e.g. cytotoxicity, mutagenesis.

v. Studies dealing with genetics e.g. genetic analysis, immortalization, senescence.

vi. Laboratory production of medical/pharmaceutical compounds for wide range of applications e.g. vaccines, interferon's, hormones.

There are however, several limitations on the use of animal cell cultures. This is mostly due to the differences that exist between the in vivo and in vitro systems, and the validity of the studies conducted in the laboratory.

Medical / Pharmaceutical Products of Animal Cell Cultures:

The most important application of animal cell cultures is the production of a wide range of commercial compounds for medical and pharmaceutical use. A selected list of animal cell culture products of commercial importance is given in Table 33.4.

TABLE 33.4 Selected examples of animal cell culture products (proteins) of medical/pharmaceutical importance

Product(s)	*Application(s)*
Vaccines	
Polio vaccines	Poliomyelitis prophylaxis
Measles vaccine	Measles prophylaxis
Rabies vaccine	Rabies prophylaxis
Malaria vaccines	Malaria prophylaxis
HIV vaccine	AIDS prophylaxis and treatment
Plasminogen activators	
Tissue-type plasminogen activator	Acute myocardial infarction, pulmonary embolism, deep vein thrombosis, acute stroke.
Urokinase-type plasminogen activator	
Recombinant plasminogen activator	
Interferons	
Interferon-α	Anticancer, immunomodulator
Interferon-β	Anticancer, antiviral
Interferon-γ	Anticancer, immunomodulator
Blood clotting factors	
Factors VII, VIII, IX and X	Hemophilia, as blood clotting agents.
Hormones	
Human growth hormone	Growth retardation in children
Somatotropin	Chronic renal insufficiency
Follicle stimulating hormone	Treatment of infertility
Human chorionic gonadotropin	Treatment of infertility
Monoclonal antibodies	
Anti-lipopolysaccharide	Treatment of sepsis
Human B-cell Lymphomas	Treatment of B-cell lymphoma
Anti-fibrin 99	Diagnosis of blood clot by imaging
Tcm-FAb (breast)	Diagnosis of breast cancer
Others	
Erythropoietin	Antianaemic agent
Interleukin-2	Anticancer, HIV treatment
Tumor necrosis factor	Anticancer
Granulocyte stimulating factor	Anticancer
Carcinoembryonic antigen	Diagnosis and monitoring of cancer patients.

Production of vaccines:

Monkey kidney or chick embryo cells or recently human diploid cells are in use for the production of vaccines. The vaccine manufacture in animal cell cultures is rather complex with risk of contamination, and safety aspect. For

these reasons, production of vaccines by recombinant DNA technology employing bacteria or yeasts is preferred.

Production of high value therapeutics:

Many human proteins with high therapeutic potential are often in short supply e.g. tissue plasminogen activator, clotting factors (VIII and IX), and erythropoietin. There is a major limitation to produce human proteins that undergo post- translational modifications (glycosylation, carboxylation etc.) in bacteria and yeasts.

This is due to the fact that these organisms do not possess the machinery to perform post-translational changes. However, pharmaceutical proteins that do not require post-translational modifications can be produced by bacteria or yeasts e.g. insulin, albumin, growth hormone. Animal cell cultures (particularly mammalian cell cultures) are useful for the production of many pharmaceutically/medically important proteins (Table 33.4).

These include the following:

i. Plasminogen

ii. Interferon

iii. Blood clotting factors

iv. Hormones

v. Monoclonal antibodies

vi. Erythropoietin

Purification of pharmaceutical products:

As the desired product is produced in the cell culture medium, its purification, isolation and storage (collectively referred to as downstream

processing) assumes significance. The final product for therapeutic applications is expected to satisfy the following criteria.

i. The product should have a stable structure with optimal activity.

ii. The product should be free from other biomolecules that may interfere with its activity and/or cause immunological complications.

iii. It should be free from all pathogens including viruses.

Genetic Engineering of Animal Cells and their Applications:

It is now possible to genetically modify the animal (mammalian) cells to introduce the genes needed for the production of a specific protein or improve the characteristics of a cell line.

The following methods are used to introduce foreign DNA into mammalian cells:

1. Electroporation

2. Lipofection

3. Microinjection

4. Fusion of mammalian cells with bacteria or viruses.

As the foreign DNA gets integrated into the mammalian cellular genome, the gene expresses to produce the desired protein. It is however, necessary to select the best producing recombinant cells by conventional methods using selectable marker genes.

The following selectable markers are used for choosing the transfected cells:

i. Viral thymidine kinase

ii. Bacterial dihydrofolate reductase

iii. Bacterial neomycin phosphotransferase

It has been possible to overproduce several proteins in mammalian cells through genetic manipulations e.g., tissue plasminogen activator, erythropoietin, interleukin-2, interferon-β, clotting factors VIII and IX, tumor necrosis factors. The recombinant mammalian cells are conveniently used for the production of monoclonal antibodies which have wide range of applications.