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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 1 – ANIMAL CELL CULTURE – SBTA 1601

ANIMAL BIOTECHNOLOGY UNIT

1 - ANIMAL CELL CULTURE - COURSE MATERIAL

Introduction, importance and history of cell culture development:

Cell culture is the growth of cells from an animal or plant in an artificial, controlled environment. Cells are removed either from the organism directly and disaggregated before cultivation or from a cell line or cell strain that has previously been established.

Certain culture conditions depend on the cell type, however, each culture must consist of a suitable vessel with a substrate or medium that supplies the nutrients (such as amino acids, carbohydrates, vitamins, minerals), growth factors or essential hormones for culturing cells. Gases (O2, CO2), physicochemical environment (pH, osmotic pressure, temperature) also play an important role to regulate the proper cell growth in an artificial environment.

Cell Culture Environment:

One of the major advantages of cell culture is the ability to manipulate the **physico-chemical** (i.e., temperature, pH, osmotic pressure, O_2 and CO_2 tension) and the **physiological environment** (i.e., hormone and nutrient concentrations) in which the cells propagate.

While the physiological environment of the culture is not as well defined as its physico-chemical environment, a better understanding of the components of serum, the identification of the growth factors necessary for proliferation, and a better appreciation of the microenvironment of cells in culture (i.e., cell-cell interactions, diffusion of gases, interactions with the matrix) now allow the culture of certain cell lines in serum-free media.

Considerations for creating an optimized cell culture environment for your cells:

pH levels

Most normal **mammalian cell lines** grow well at pH 7.4, and there is very little variability among different cell strains. However, some transformed cell lines have been shown to grow better at slightly more acidic environments (pH 7.0 – 7.4), and some normal fibroblast cell lines prefer slightly more basic environments (pH 7.4 – 7.7). **Insect cell lines** such as Sf9 and Sf21 grow optimally at pH 6.2.

CO_2 levels

The growth medium controls the pH of the culture and buffers the cells in culture against changes in the pH. Usually, this buffering is achieved by including an organic (e.g., HEPES) or CO_2-bicarbonate based buffer. Because the pH of the medium is dependent on the delicate balance of dissolved carbon dioxide (CO_2) and bicarbonate (HCO_3^-), changes in the atmospheric CO_2 can alter the pH of the medium. Therefore, it is necessary to use exogenous CO_2 when

using media buffered with a CO_2-bicarbonate based buffer, especially if the cells are cultured in open dishes or transformed cell lines are cultured at high concentrations. While most researchers usually use 5 – 7% CO_2 in air, 4 – 10% CO_2 is common for most cell culture experiments. However, each medium has a recommended CO_2 tension and bicarbonate concentration to achieve the correct pH and osmolality.

Optimal temperatures for various cell lines:

The optimal temperature for cell culture largely depends on the body temperature of the host from which the cells were isolated, and to a lesser degree on the anatomical variation in temperature (e.g., temperature of the skin may be lower than the temperature of skeletal muscle). Overheating is a more serious problem than underheating for cell cultures; therefore, often the temperature in the incubator is set slightly lower than the optimal temperature.

- Most **human and mammalian cell lines** are maintained at 36°C to 37°C for optimal growth.
- **Insect cells** are cultured at 27°C for optimal growth; they grow more slowly at lower temperatures and at temperatures between 27°C and 30°C. Above 30°C, the viability of insect cells decreases, and the cells do not recover even after they are returned to 27°C.
- **Avian cell lines** require 38.5°C for maximum growth. Although these cells can also be maintained at 37°C, they will grow more slowly.
- Cell lines derived from **cold-blooded animals** (e.g., amphibians, cold-water fish) tolerate a wide temperature range between 15°C and 26°C.

Cell Culture Equipment:

The specific requirements of a cell culture laboratory depend mainly on the type of research conducted; for example, the needs of mammalian cell culture laboratory specializing in cancer research is quite different from that of an insect cell culture laboratory that focuses on protein expression. However, all cell culture laboratories have the common requirement of being free from pathogenic microorganisms (i.e., asepsis), and share some of the same basic equipment that is essential for culturing cells.

1. Incubators

The purpose of the incubator is to provide the appropriate environment for cell growth. The incubator should be large enough for your laboratory needs, have forced-air

circulation, and should have temperature control to within ±0.2°C. Stainless steel incubators allow easy cleaning and provide corrosion protection, especially if humid air is required for incubation. Although the requirement for aseptic conditions in a cell

culture incubator is not as stringent as that in a cell culture hood, frequent cleaning of the incubator is essential to avoid contamination of cell cultures.

Types of Incubators

There are two basic types of incubators, dry incubators and humid CO_2 incubators. **Dry incubators** are more economical, but require the cell cultures to be incubated in sealed flasks to prevent evaporation. Placing a water dish in a dry incubator can provide some humidity, but they do not allow precise control of atmospheric conditions in the incubator. **Humid CO_2 incubators** are more expensive, but allow superior control of culture conditions. They can be used to incubate cells cultured in Petri dishes or multiwell plates, which require a controlled atmosphere of high humidity and increased CO_2 tension.

2. Storage

A cell culture laboratory should have storage areas for liquids such as media and reagents, for chemicals such as drugs and antibiotics, for consumables such as disposable pipettes, culture vessels, and gloves, for glassware such as media bottles and glass pipettes, for specialized equipment, and for tissues and cells. Glassware, plastics, and specialized equipment can be stored at ambient temperature on shelves and in drawers; however, it is important to store all media, reagents, and chemicals according to the instructions on the label.

Some media, reagents, and chemicals are sensitive to light; while their normal laboratory use under lighted conditions is tolerated, they should be stored in the dark or wrapped in aluminum foil when not in use.

3. Refrigerators

For small cell culture laboratories, a domestic refrigerator (preferably one without an autodefrost freezer) is an adequate and inexpensive piece of equipment for storing reagents and media at 2–8°C. For larger laboratories, a cold room restricted to cell culture is more appropriate. Make sure that the refrigerator or the cold room is cleaned regularly to avoid contamination.

4. Freezers

Most cell culture reagents can be stored at –5°C to –20°C; therefore an ultradeep freezer (i.e., a –80°C freezer) is optional for storing most reagents. A domestic freezer is a cheaper alternative to a laboratory freezer. While most reagents can withstand temperature oscillations in an autodefrost (i.e., self-thawing) freezer, some reagents such as antibiotics and enzymes should be stored in a freezer that does not autodefrost.

5. Cryogenic Storage

Cell lines in continuous culture are likely to suffer from genetic instability as their passage number increases; therefore, it is essential to prepare working stocks of the cells and preserve them in cryogenic storage (for more information, see **Freezing Cells**). **Do not store cells in –20°C or –80°C freezers**, because their viability quickly decreases when they are stored at these temperatures.

There are two main types of liquid-nitrogen storage systems, **vapor phase** and liquid phase, which come as wide-necked or narrow-necked storage containers. Vapor phase systems minimize the risk of explosion with cryostorage tubes, and are required for storing biohazardous materials, while the **liquid phase** systems usually have longer static holding times, and are therefore more economical.

Narrow-necked containers have a slower nitrogen evaporation rate and are more economical, but **wide-necked** containers allow easier access and have a larger storage capacity.

6. Cell Counter

A cell counter is essential for quantitative growth kinetics, and a great advantage when more than two or three cell lines are cultured in the laboratory.

<u>History of Animal Cell Culture:</u>

1878:

Claude Bernard proposed that physiological systems of an organism can be maintained in a living system after the death of an organism.

1885:

Roux maintained embryonic chick cells in a saline culture.

1897:

Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.

1903:

Jolly observed cell division of salamander leucocytes in vitro.

1907:

Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibres in vitro for several weeks. He was considered by some as the father of cell culture.

1910:

Burrows succeeded in long-term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.

1911:

Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.

1913:

Carrel introduced strict aseptic techniques so that cells could be cultured for long periods.

1916:

Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.

1923:

Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.

1927:

Carrel and Rivera produced the first viral vaccine – Vaccinia.

1933:

Gey developed the roller tube technique.

1940s:

The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture.

1948:

Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium, CMRL 1066.

1949:

Enders reported that polio virus could be grown on human embryonic cells in culture.

1952:

Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.

1954:

Abercrombie observed contract inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.

1955:

Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.

1961:

Hay flick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite life-span in culture.

1964:

Littlefield introduced the HAT medium for cell selection.

1965:

Ham introduced the first serum-free medium which was able to support the growth of some cells.

1965:

Harris and Watkins were able to fuse human and mouse cells by the use of a virus.

1975:

Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody.

1978:

Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.

1982:

Human insulin became the first recombinant protein to be licensed as a therapeutic agent.

1985:

Human growth hormones produced from recombinant bacteria was accepted for therapeutic use.

1986:

Lymphoblastoidy γlFN licensed.

1987:

Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.

1989:

Recombinant erythropoietin in trial.

1990:

Recombinant products in clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).

Basic Tissue Culture techniques:

Tissue culture is a method of biological research in which fragments of tissue from an animal or plant are transferred to an artificial environment in which they can continue to survive and function. The cultured tissue may consist of a single cell, a population of cells, or a whole or part of an organ. Cells in culture may multiply; change size, form, or function; exhibit specialized activity (muscle cells, for example, may contract); or interact with other cells.

Suspension Cultures

Suspension cultures are generally used for anchorage-independent cells, such as hemopoietic cells and some transformed cell lines, and for free-floating cells or cell formations (e.g., isolated cells, aggregates, or tissue fragments). Anchorage-independent cells are grown in either a semisolid medium, such as agarose, or in fluid suspension culture under continuous agitation (stirring or shaking). Short-term suspension cultures of bulk, isolated brain cells have also been described (Verity, 1995). In general, suspension cultures are easy to maintain and are ideal for scale-up. For the latter, culture vessels of various sizes are available, ranging from small spinner flasks to industrial-size fermenters (e.g., Jakoby and Pastan, 1979).

Attached ("Monolayer") Cell Culture

Monolayer cell culture is the most widely used technique, with numerous protocol variants of this approach practiced in different laboratories (Sensenbrenner, 1977; Conn, 1990; Jakoby and Pastan, 1979). In these cultures, the cells grow attached to the surface of the culture vessel, although specific treatment of the culture vessel surface (e.g., with polylysine or polyornithine) may be necessary for better cell Supplement 4 Current Protocols in Pharmacology 12.1.2 Overview of Cell and Tissue Culture Techniques attachment and/or attainment of more physiological growth conditions. The growth conditions are also influenced by the cell density. Techniques using different cell densities range from single-cell microcultures to high-density mass cell cultures. The yield in monolayer cultures is limited by the available surface to which the cells may attach. Therefore, for scaling up, cells may be grown in roller bottles (cylindrical bottles partially filled with medium and rotated around their horizontal axis at 40 to 50 rpm to provide continuous medium supply), in capillary perfusion systems (in which medium is circulated through tightly packed artificial capillaries), or attached to particulate microcarriers that can be maintained in suspension culture (Conn, 1990). With a high-celldensity culture, essential metabolites may be rapidly depleted, causing density-dependent growth inhibition and eventually cell death. One advantage of monolayer cultures is their ready accessibility for direct microscopic examination, as well as for morphological, immunocytochemical, and electrophysiological studies. On the other hand, it is relatively difficult to sample monolayer cultures for ultrastructural or biochemical analyses without destroying the culture. Furthermore, with these cultures, the cells are attached to an artificial substrate rather than to the natural extracellular matrix, limiting direct cell-cell interactions.

Three-Dimensional (Aggregate) Cell Cultures

Aggregate cultures are prepared from dissociated cells allowed to reaggregate under controlled conditions and continuous gyratory agitation to form regular, spherical cell structures. Such cultures permit a maximum of cell-cell interactions and thus the development of a natural cell matrix and histotypic cell formations. Aggregate cultures prepared from fetal cells show a particularly high capacity for cellular reorganization and maturation. Although it has been shown by Moscona, who invented this particular cell culture technique (Moscona, 1965), that immature cells of any tissue are able to reaggregate and mature into histotypic structures, this method is most suitable for immature neural cells (Conn, 1990; Fedoroff and Richardson, 1997). One advantage of aggregate cultures is that they provide large numbers of highly reproducible replicates, from which aliquots are readily sampled for multidisciplinary studies. On the other hand, aggregate cultures do not permit the direct microscopic observation of cells during culture. Furthermore, with three-dimensional cultures, oxygen and nutritional gradients are critical, limiting the size of the individual aggregate to a diameter less than 400 μm. Moreover, in certain preparations, such as aggregate cultures of fetal liver cells, an additional diffusion barrier is formed by a peripheral epitheloid cell layer. This problem is not encountered with aggregate cultures of fetal brain cells, which can be maintained for months at a highly differentiated stage. Tumor cells or explants grown in sponge-gel matrices form three-dimensional structures (called spheroids) and exhibit tissue-specific drug responses (Celis, 1998).

Explant Cultures

Explant cultures are prepared either from intact organs, such as dorsal root ganglia, sympathetic ganglia, parasympathetic ciliary ganglia, or from small tissue fragments that do not exceed 1 mm in any dimension. This approach is particularly useful for neural tissues which, due to their structural and functional complexity, are most difficult to reconstitute in vitro. Explants may be maintained in stationary culture on coverglasses or coverslips (Crain, 1976; Bornstein, 1995; Fedoroff and Richardson, 1997), or they may be placed on porous and transparent membranes (e.g., Millipore Millicell-CM; Falcon Cyclopore; Whatman Anodisc) that remain in contact with liquid medium in such a way that a thin film of liquid surrounds the tissue (Stoppini et al., 1991). Often, the attachment of explants is enhanced by substrate coating using collagen, laminin, or polylysine. As a further variant, brain slices embedded in a plasma clot on glass coverslips are maintained in a roller tube, which allows for alternating exposure of the tissue to air and liquid medium (Gähwiler, 1981). Explants (or microexplants from minced tissue) may also be maintained in nonadherent organ culture by using continuous gyratory agitation to keep the tissue in suspension. Stationary as well as free-floating explants preserve their cytoarchitectonic and gross anatomic cellular organization to a large extent, although structural and functional abnormalities relative to the in vivo situation occur. Roller tube slice cultures decrease in thickness to one to three cell layers within a few days of culture, but generally retain their histotypic organization. This preparation is particularly well suited for direct microscopic observation and electrophysiological studies. As a rule, explant cultures are the best approximation of the in vivo situation with respect to the organ-specific cellular organization. For example, cerebellar explants develop normal architectural arrangements of cortical laminae and deep nuclei, and hippocampal explants form functional synaptic networks in culture, as well as normal dendritic arborizations of pyramidal and granule cells. Explant cultures also offer a unique system for the study of adjacent brain regions in coculture, such that they are able to form interacting afferent and efferent projections under in vitro conditions (Fedoroff and Richardson, 1997). The main disadvantages of explant cultures are the small amount of tissue available and the relatively low number and limited reproducibility of replicate cultures, making it difficult to use them for biochemical and molecular biology studies.

Media used for Animal cell culture

In animal tissue culture, 2 types of culture media are used:

Natural media

Artificial media

The type of medium relies basically on the type of cells to be cultured and its objectives.

1. Natural media:

These media include the naturally occurring biological fluids and are of the following three types:

Clots

Biological fluids

Tissue extract

i) Clots:

Plasma clots are the most commonly used clots and has been employed for a long time.

In the present time, plasma is commercially found in liquid state that can be prepared in the laboratory. ii) Biological Fluids:

Several biological fluids can be employed as culture media such as amniotic fluid, pleural and ascetic fluids, hemolymph of insects, aqueous humoral from eye, serum etc.

Among them, serum is the mostly preferred.

iii) Tissue Extracts:

The most commonly used tissue extract is chick embryo extract, however, bovine embryo extract is also used.

In the culture media, the extracts from spleen, liver, bone marrow and leucocytes were also used.

The substitution for tissue extract can be a mixture of amino acids and certain other organic compounds.

2. Artificial media:

For the following purposes, various artificial media have been employed:

- Immediate survival (a balanced salt solution with specified pH and adequate osmotic pressure)
- Prolonged survival (a balanced salt solution in addition with serum, or appropriate formulation of organic compounds.
- Indefinite growth
- Specialized functions
- Artificial media may be classified into following types:
- Serum containing media
- Serum-free media
- Protein free media
- Chemically defined media

Serum:

Serum is the yellowish liquid and is a transparent content that remains left over after the removal of fibrin and cells from the blood.

2-10% of serum is often contained by normal growth media.

The most commonly employed supplement in animal cell culture is fetal bovine serum (FBS).

It supplies the basic nutrients for cells.

It also contains several hormones and various growth factor.

In addition to it, it also acts as a buffer.

Serum containing media:

In animal cell culture media, fetal bovine serum is the most common supplement.

In order to supply an optimal culture medium, it is employed as an economical supplement.

Serum supplies carriers water-insoluble nutrients, protease inhibitors, hormones and growth factors and binds and neutralizes the toxic moieties.

Serum free media:

In case of immunological studies, presence of serum in media can result to serious misinterpretations.

In general, these media are specifically designed to promote the culture of a single type of cell and incorporate specified amounts of purified growth factors, lipoproteins and other proteins normally supplied by the serum.

As the components of these media are known, thus it is referred to as 'defined culture media'
Chemically defined media:

These media contain ultra-pure inorganic and organic ingredients free of contaminants and may also contain pure protein additives, such as growth factors.

Their constituents are produced by genetic modification in bacteria or yeast with the addition of vitamins, cholesterol, particular amino acids, and fatty acids.

Protein free media:

Protein-free media is devoid of any protein and only include non-protein constituents.

Usage of protein-free media enhances superior cell growth and protein expression in contrast to serum-supplemented media and enables downstream purification of any expressed product.

In order to choose a medium, the minimum criteria required usually involve:

- The medium should supply the cells with all of the nutrients.
- Keep the physiological pH about 7.0 with ample buffering.
- The medium should be sterile, and isotonic to the cells.

- The balanced salt solution that was initially used to establish a physiological pH and osmolarity needed to sustain cells in vitro was the basis for the cell culture media.
- Various components (glucose, amino acids, vitamins, growth factors, antibiotics etc have been added to promote cell growth and proliferation, and several media have been created.

Primary vs secondary cell cultures, their Maintenance and Preservation

A. Primary cell culture

This is the cell culture obtained straight from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture. Such culture comprises mostly heterogeneous cells and most of the cells divide only for a limited time. However, these cells are much similar to their parents.

Depending on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent cells

These cells are anchorage dependent and propagate as a monolayer. These cells need to be attached to a solid or semi-solid substrate for proliferation. These adhere to the culture vessel with the use of an extracellular matrix which is generally derived from tissues of organs that are immobile and embedded in a network of connective tissue. Fibroblasts and epithelial cells are of such types.

When the bottom of the culture vessel is covered with a continuous layer of cells, usually one cell in thickness, these are known as monolayer cultures. Majority of continuous cell lines grow as monolayers. As being single layers, such cells can be transferred directly to a cover slip to examine under a microscope.

Suspension cells

Suspension cells do not attach to the surface of the culture vessels. These cells are also called anchorage independent or non-adherent cells which can be grown floating in the culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells can be grown in suspension. These cells grow much faster which do not require the frequent replacement of the medium and can be easily maintained. These are of homogeneous types and enzyme treatment is not required for the dissociation of cells; similarly these cultures have short lag period.

Confluent culture and the necessity of sub-culture

After the cells are isolated from the tissue and proliferated under the appropriate conditions, they occupy all of the available substrate i.e. reach confluence. For a few days, it can become too crowded for their container and this can be detrimental to their growth, generally leading to cell death if left for a long time. The cells thus have to be subculture i.e. a portion of cells is transferred to a new vessel with fresh growth medium which provides more space and nutrients for the continual growth of both portions of cells. Hence subculture keeps cells healthy and in a growing state.

A passage number refers specifically to how many times a cell line has been sub-cultured. In contrast with the population doubling level in that the specific number of cells involved is not relevant. It simply gives a general indication of how old the cells may be for various assays.

B. Secondary cell culture and cell line

When a primary culture is sub-cultured, it is known as secondary culture or cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically).

Sub-culturing of primary cells to different divisions leads to the generation of cell lines. During the passage, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. However, as they are sub-cultured serially, they become different from the original cell.

On the basis of the life span of culture, the cell lines are categorized into two types:

Finite cell lines

The cell lines which go through a limited number of cell division having a limited life span are known as finite cell lines. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

Continuous cell lines

When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. Such transformation or mutation can occur spontaneously or can be chemically or virally induced or from the establishment of cell cultures from malignant tissue. Cell cultures prepared in this way can be sub-cultured and grown indefinitely as permanent cell lines and are immortal.

These cells are less adherent, fast growing, less fastidious in their nutritional requirements, able to grow up to higher cell density and different in phenotypes from the original tissue. Such cells grow more in suspension. They also have a tendency to grow on top of each other in multilayers on culture-vessel surfaces.

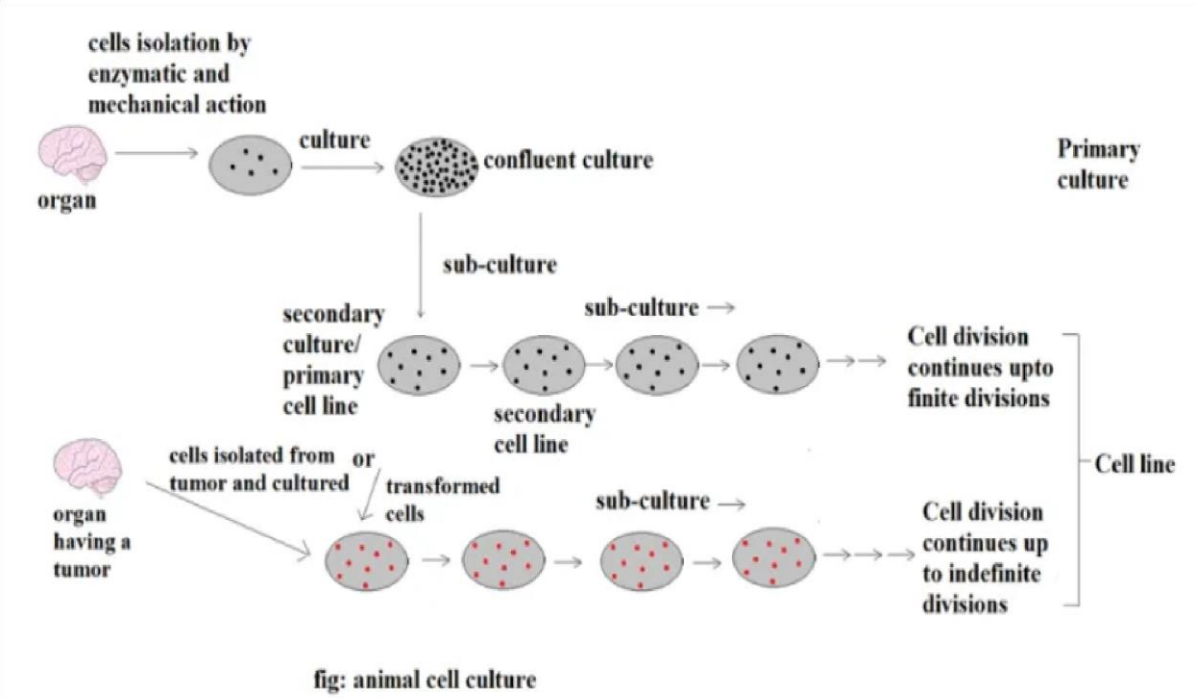

Figure 1: Animal Cell Culture

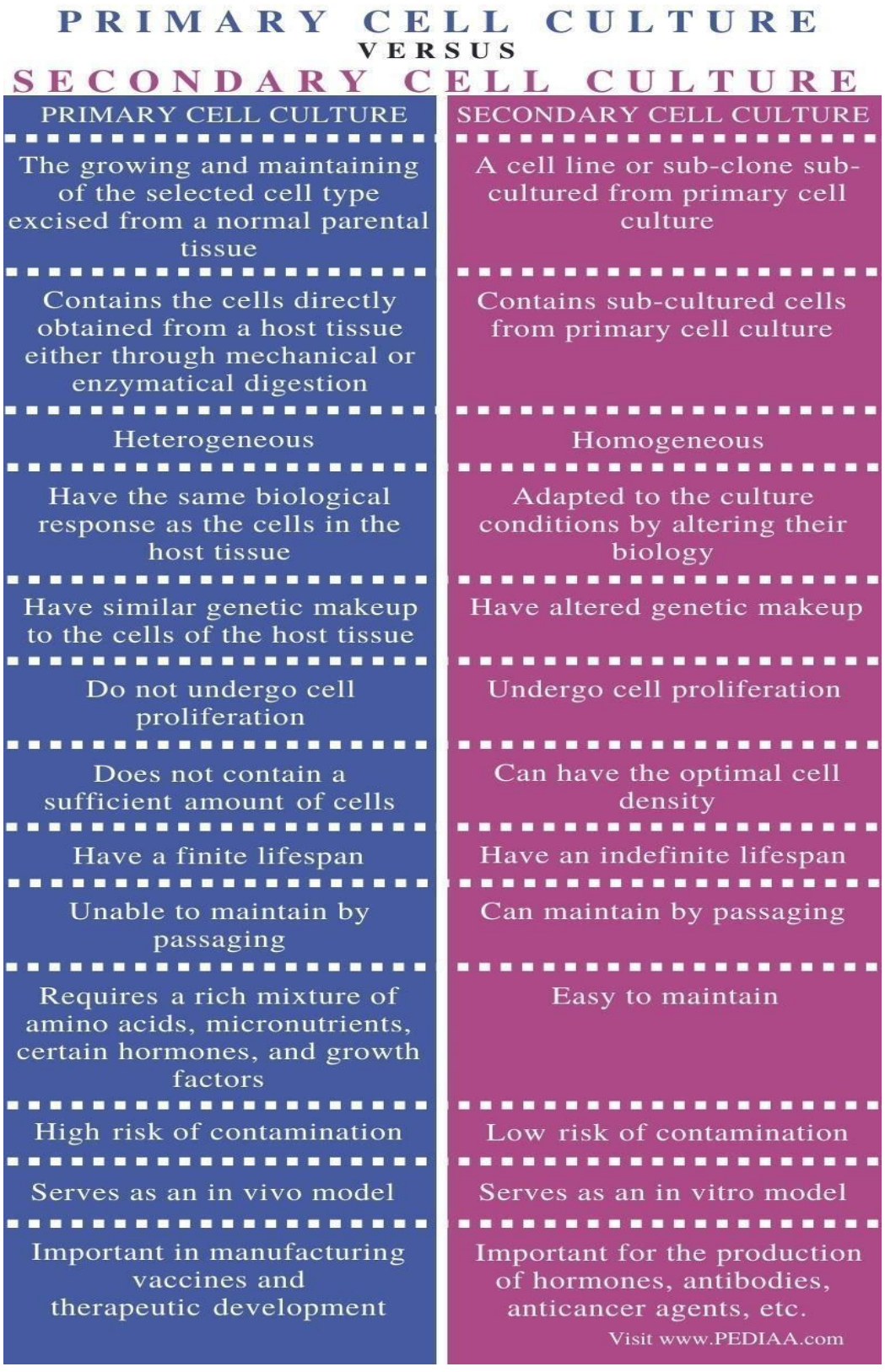

Table 1: Primary Cell Culture vs Secondary Cell Culture

CULTURE MAINTENANCE

Today a variety of ways are available to cell culture incubators, vessels, and antibiotics that allow tightly conditions for culture to avoid contamination and infection. but sometimes require to maintain to culture for a long time for this the best method called cryo-preservation or frizzing of culture by the use of cryoprotectant DMSO(dimethyl sulfoxide) that is a toxic agent. in this process cells preserve at very low temperature and on this cell tolerate to very low metabolic activity.

Equipment

- laminar air flow hood.
- mechanical pipetter.
- inverted microscope.
- vacuum pump and flask. - Co2 incubator.

Process- (Cell Culture Protocol)

- use only healthy cells or tissues.
- detach cells with trypsin.
- than resuspend cells to the growth medium.
- adjust concentration about 4–8 x 106 cells/ml.
- than filled to culture in specific vials or ampules.
- then freeze them slowly.
- then transfer to vials in gaseous phase mean at liquid nitrogen (-196°C) with liquid nitrogen.

Recovery of preserved cells-

In this process, preserved ampules transfer on a water bath at 36°C. then transfer to cells on appropriate medium and allow to produce cell monolayer.

Applications of Animal cell culture

A. Vaccines Production

One of the most important uses of cell culture is in research and production of vaccines. The ability to grow large amounts of virus in cell culture eventually led to the creation of the polio vaccine, and cells are still used today on a large scale to produce vaccines for many other diseases, like rabies, chickenpox, hepatitis B, and measles. In early times, researchers had to use live animals to grow poliovirus, but due to the development of cell culture techniques, they were able to achieve much greater control over virus production and on a much larger scale which eventually develop vaccines and various treatments. However, continuous cell lines are not used in virus production for human vaccines as these are derived from malignant tissue or possess malignant characteristics.

B. Virus cultivation and study

Cell culture is widely used for the propagation of viruses as it is convenient, economic, easy to handle compared to other animals. It is easy to observe cytopathic effects and easy to select particular cells on which the virus grow as well as to study the infectious cycle. Cell lines are convenient for virus research because cell material is continuously available. Continuous cell lines have been extremely useful in cultivating many viruses previously difficult or impossible to grow.

C. Cellular and molecular biology

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of different toxic compounds on the cells, and mutagenesis and carcinogenesis. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

D. In Cancer Research

Normal cells can be transformed into cancer cells by methods including radiation, chemicals, and viruses. These cells can then be used to study cancer more closely and to test potential new treatments.

E. Gene therapy

Cells having a functional gene can be replaced to cells which are having non-functional gene, and for which the cell culture technique is used.

F. Immunological studies

Cell culture techniques are used to know the working of various immune cells, cytokines, lymphoid cells, and interaction between disease-causing agents and the host cells.

G. Others

Cell lines are also used in in-vitro fertilization (IVF) technology, recombinant protein, and drug selection and improvement.

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UNIT – 2 – GROWTH AND SCALE UP – SBTA 1601

Cell growth and growth kinetics of animal cells

Cell growth refers to the increase in cell size (mass accumulation) while cell division describes the division of a mother cell into two daughter cells (1->2->4->8, etc.). Cell proliferation is the process of

In animals, the growth of animals is more restricted in time than is that of plants, but cell division is more generally distributed throughout the body of the organism. Although the rate of cell division differs in different regions, the capacity for cell division is widely distributed in the developing embryo generating an increased number of cells through cell division. Plants differ from animals in their manner of growth. As young animals mature, all parts of their bodies grow until they reach a genetically determined size for each species. Plant growth, on the other hand, continues throughout the life span of the plant and is restricted to certain meristematic tissue regions only.

Growth kinetics is an autocatalytic reaction which implies that the rate of growth is directly proportional to the concentration of cell. The cell concentration is measured by direct and indirect methods. Direct methods include measuring the cell mass concentration and cell number density by its dry weight, turbidity (optical density), plate counts etc. Whereas, indirect methods of measuring cell density are done by measuring the concentration of proteins, ATP or DNA content.

Batch growth kinetics of a microbe follows a growth curve with lag phase as the initial phase during which cells adapt to a new environment. Multiple lag phases occur if the media is supplemented with more than one sugar and such type of growth is referred to as diauxic growth. Following the lag phase is the log phase in which the cell mass and cell number increases exponentially and then the depletion of nutrients starts which indicates the deceleration phase. The accumulation of toxic products results in deceleration phase after which stationary phase commences in which growth rate equals the death rate. The continuous growth kinetics accessed by a perpetual feeding process in which the growth is controlled by the concentration of the rate limiting nutrient.

1. Lag Phase-The single cell inoculation into the liquid media doesn’t start dividing as per its generation time. During this phase bacteria gets adjusted to the new media and grow in size instead of dividing into daughter cells. In this phase, bacteria synthesize the most crucial enzymes or co-enzyme present in traces and required for optimal growth and multiplication. In addition, cell is metabolically active and be busy in synthesizing large amount of protoplasm. At the end of this phase, each bacterial cell divides and enter into the next phase of active multiplication

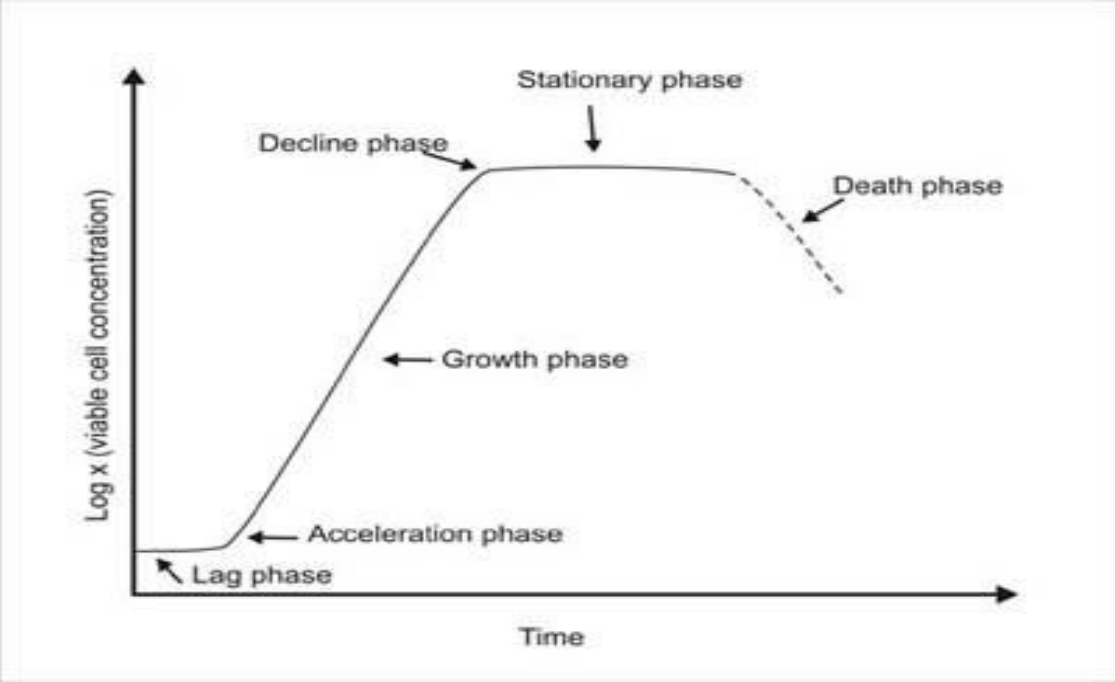


Figure 2: Typical growth curve for a batch system

2. Log Phase-In this phase, bacterial cell population is involved in active division and whole cellpopulation is more or less homogenous in terms of chemical composition, physiology and metabolic activity. A plot of number of cell (in log scale) against time gives straight line. The growth of bacterial cell population is increasing at a constant rate and continues until substrate concentration is not limiting.
3. Stationary Phase-Once substrate is limiting, the logarithmic phase of growth begins to declinegradually with a constant number of cells to give a straight line. The population remains constant because number of divisions are equal to the number of death events. As substrate is limiting, death of old cell provides enough nutrient for remaining cells to grow and multiply to maintain the constant number.
4. Death Phase-When substrate is not sufficient from dying cells, death rate of bacteria superseedrate of growth and as a result number of bacteria declines sharply.

The Monod equation is a mathematical model for the growth of microorganisms. It is named for Jacques Monod (1910 – 1976, a French biochemist, Nobel Prize in Physiology or Medicine in 1965), who proposed using an equation of this form to relate microbial growth rates in an aqueous environment to the concentration of a limiting nutrient. The Monod equation has the same form as the Michaelis–Menten equation, but differs in that it is empirical while the latter is based on theoretical considerations.

The empirical Monod equation is:

$$\mu = \mu_{\max} \frac{[S]}{K_s + [S]}$$

where:

- μ is the growth rate of a considered microorganism
- μ_{max} is the maximum growth rate of this microorganism
- [S] is the concentration of the limiting substrate S for growth
- Ks is the "half-velocity constant"—the value of [S] when $\mu/\mu_{max} = 0.5$

μ_{max} and Ks are empirical (experimental) coefficients to the Monod equation. They will differ between microorganism species and will also depend on the ambient environmental conditions, e.g., on the temperature, on the pH of the solution, and on the composition of the culture medium.

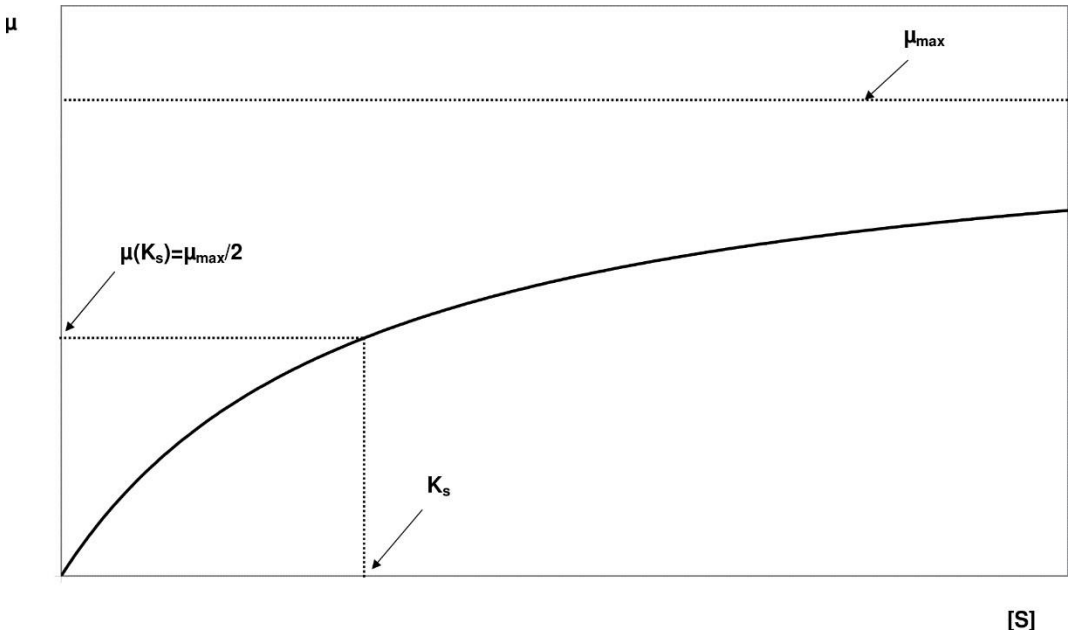


Figure 2.1: The growth rate μ of a considered microorganism as a function of the limiting substrate concentration [S]

Various Types of Cultures microcarrier attached growth, suspension Cultures, Continuous Flow Cultures

Modifying a laboratory procedure, so that it can be used on an industrial scale is called scaling up. Laboratory procedures are normally scaled up via intermediate models of increasing size. The larger the plant, the greater the running costs, as skilled people are required to monitor and maintain the machinery. The first pre-requisite for any large-scale cell culture

system and its scaling up is the establishment of a cell bank. Master cell banks (MCB) are first established and they are used to develop Master Working Cell Banks (MWCB). The MWCB should be sufficient to feed the production system at a particular scale for the predicted life of the product. The cell stability is an important criterion so MWCB needs to be repeatedly sub cultured and each generation should be checked for changes. A close attention should be paid to the volume of cultured cells as the volume should be large enough to produce a product in amounts which is economically viable. The volume is maintained by

(a) increasing the culture volume,

(b) by increasing the concentration of cells in a reactor by continuous perfusion of fresh medium,so that the cells keep on increasing in number without the dilution of the medium.

A fully automated bioreactor maintains the physicochemical and biological factors to optimum level and maintains the cells in suspension medium. The most suitable bioreactor used is a compact-loop bioreactor consisting of marine impellers. The animal cells unlike bacterial cells, grow very slowly. The main carbon and energy sources are glucose and glutamine. Lactate and ammonia are their metabolic products that affect growth and productivity of cells. So, the on-line monitoring of glucose, glutamate, and ammonia is carried out by on line flow injection analysis (FIA) using gas chromatography (GC), high performance liquid chromatography (HPLC) etc .

Cell cultures are used for obtaining useful products like bio-chemicals (interferon, interleukins, hormones, enzymes, antibodies, etc.) and virus vaccines (polio, mumps, measles, rabies, foot and mouth, rinderpest etc.).For these objectives, large scale cell cultures are essential; fermenters of 5,000 to 20,000 L are used for this purpose.

The scaling up of cell cultures may be done as follows:

(1) as monolayer cultures,

(2) as suspension cultures, or

(3) as immobilized cell systems.

For obvious reasons, scaling up of monolayer systems is more difficult than that of others.

1. **Monolayer Culture:**

   Monolayer cultures are essential for anchorage dependent cells. Scaling up of such cultures is based on increasing the available surface area by using plates, spirals, ceramics and micro-carriers (most effective). The various culture vessels used are briefly described below.

   Roux Bottle: It is commonly used in laboratory, and is kept stationary so that only a portion of its internal surface is available for cell anchorage . Each bottle provides Ca. 175- 200 cm2 surface area for cell attachment and occupies 750-1000 cm3 space.

Roller Bottle: This vessel permits a limited scale up as it is rocked or preferably, rolled so that its entire internal surface is available for anchorage. Several modifications of roller bottle further enhance the available surface, e.g., (i) Spira-Cel (spiral polystyrene cartridge), (ii) glass tube (roller bottle packed with a parallel cluster of small glass tubes separated by silicone spacer rings), and (iii) extended surface area roller bottle (the bottle surface is corrugated enhancing the surface by a factor of two), etc.

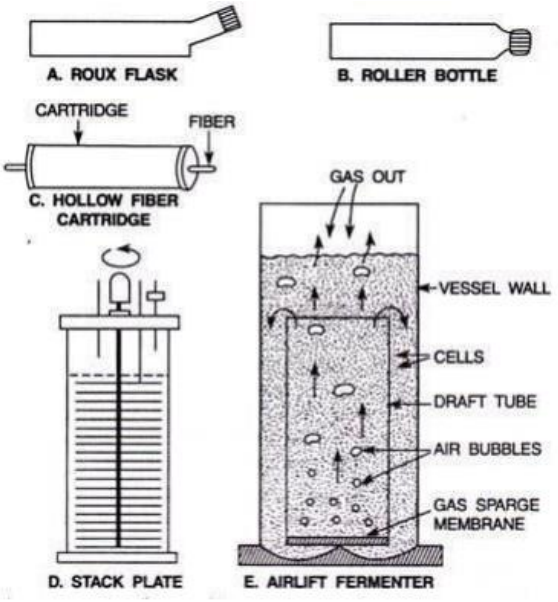


Figure 2.2: A schematic representation of some of the various types of culture vessels used for scaling up of animal cell cultures. A. Roux flask, B. Roller bottle, C. Hollow fiber cartridge. D. Stack plate vessel, and E. Airlift fermentor.

Multitray Unit: A standard unit has 10 chambers stacked on each other, which have interconnecting channels; this enables the various operations to be carried out in one go for all the chambers. Each chamber has a surface area of 600 cm2 and the total volume of the unit is 12.5 1. This polystyrene unit is disposable and gives good results similar to plastic flasks.

Synthetic Hollow Fibre Cartridge: The fibres enclosed in a sealed cartridge provide a large surface area for cell attachment on the outside surface of fibres. The capillary fibres are made up of acrylic polymer, are 350 µm in diameter with 75 µm thick walls. The medium is pumped in through the fiber; it perfuses through the fiber walls and becomes available to the cells. The surface area available is very high (upto 30 cm2 /ml of medium volume). The system is mainly used for suspension cells, but is also suitable for cell anchorage if polysulphone type fiber is used.

Opticell Culture System: It consists of a cylindrical ceramic cartridge in which 1 mm2 channels run through the length of the unit, and perfusion loop to a reservoir is provided for environmental (medium, gas, etc.) control. It gives about 40 cm2 surface area/ml of medium. It is suitable for virus, cell surface antigen and monoclonal antibody production, and for both suspension and monolayer cell cultures.

Plastic Film: Teflon (fluoroethylenepropylene copolymer) is biologically inert and highly permeable to gas. Teflon bags (5 x 30 cm) filled with cells and medium (2-10 mm deep) serve as good culture vessels; cells attach to the inside surface of bags. Alternatively, teflon tubes are wrapped round a reel with a spacer and the medium is pumped through the tube; cells grow on the inside surface of tube (a culture vessel called stericell is available).

Heli-Cell vessels: These vessels are packed with polystyrene ribbons (3 mm x 5-10 mm x 100 µm) that are twisted in helical shape. The medium is pumped through the vessel, the helical shape of ribbons ensuring good circulation; the cells adhere to the ribbon surface All the culture vessels, in addition to the increased surface area due to the vessel design, allow further scaling up by the use of multiple units of the vessels. In contrast, the following three culture systems allow scaling up in a single unit by increasing the vessel volume. In addition, they make the monolayer culture system considerably similar to suspension cultures.

Bead Bed Reactors: These reactors are packed with 3-5 mm glass beads (which provide the surface for cell attachment) and the medium is pumped either up or down the bead column. Use of 5 mm beads gives better cell yields than that of 3 mm beads.

Heterogeneous Reactors: These reactors contain circular glass or stainless steel plates stacked 5-7 mm apart and fitted to a central shaft . Either an airlift pump is used for mixing or the shaft is rotated either vertically or horizontally. The chief disadvantage of the system is very low ratio of surface area to medium volume (1-2 cm2 /ml).

Microcarrier cultures: These systems use 90-300 pm dia particles as substrate for cell attachment. Initially, Dextran beads (Sephadex A-50) were used by Van Wezel in 1967; these were not entirely satisfactory due to the unsuitable charge of beads and possibly due to toxic effects.

The microcarriers available for use at present range from Dextran, polystyrene, polyacrolein, glass, poly- acrylamide, silica, DEAE sephadex, cellulose, gelatin to collagen; the specific gravity of microcarriers ranges from 1.02 to 1.05. Microcarriers greatly increase the surface area for cell attachment per unit medium volume, which can be upto 90,000 cm2 / L depending on the size and density of the beads.

Microcarrier cultures are initiated by harvesting cells from 3 L of a

logarithmic phase (log phase) culture and inoculating them in 1 L of fresh medium to which 2-3 g/L of microcarriers is then added. The culture is stirred at 15-25 rpm (revolutions per minute) for 3-8 hours. During this period, cells attach to microcarrier beads and later grow as a monolayer. The volume of culture is slowly increased to 3 1 and stirring is enhanced to the normal rates (20- 100 rpm). As the cells grow, the beads become heavier and need to be agitated at higher speeds. The medium needs to be changed every 3 days. Samples of beads can be drawn for observations on cell morphology, growth and number.

Use of microcarriers permits the handling of monolayer systems as suspension cultures. However, cells do not grow to the same degree as they do in stationary cultures. Harvesting of cells from microcarrier beads in rather simple.Stirring is stopped, the medium is drained off, the beads are washed in buffer, treated with trypsin or some other suitable enzyme, the culture is shaken at 75-125 rpm for 20-30 min, stirring is stopped for 2 min and the supernatant is poured and collected.

Alternatively, the beads may be dissolved where possible, e.g., gelatin beads are dissolved by trypsin, collagen-coated beads are treated with collagenase and dextranase is used for dextran beads; these treatments leave the cells free, which are collected.

Scaling up of microcarrier cultures can be done either by increasing the concentration of beads or by enlarging the culture vessel. When high microcarrier concentrations are used, medium perfusion becomes necessary, and efficient filters must be used to allow medium withdrawal without cells and microcarriers. The oxygen supply is problematic; it can be based on surface aeration, increased perfusion rate of fully aerated medium, and sparging into the filter compartment.

2. Suspension Cultures:

In scaling up, both chemical (O2, pH, medium constituents and removal of wastes) and physical (the configuration of bioreactor and power supplied to the reactor) factors have to be optimised for good results. The medium must be suitably stirred to keep the cells in suspension and to make the culture homogeneous; it becomes increasingly difficult with the scaling up.

Various types of stirrers range from simple magnetic stirrers, flat blade turbine impellers, to marine impellers, to those using pneumatic energy, e.g., airlift fermenter, and those using hydraulic energy, e.g., medium perfusion. Improved mixing can be obtained by changing the design of stirrer paddle or by using multiple impellers.

The objective of stirring is to achieve good mixing without causing damage to the cells. Vibro-mixer achieves stirring by vertical reciprocating motion of 0.1-S mm at a

frequency of 50 cycles/sec of a mixing disc fixed horizontally to the agitator shaft. These stirrers cause random mixing, less foaming and lower shear forces.

It is important to supply sufficient O2 without damaging the cells. Mean 02 utilization rate by cells is about 6 mg O2/106 cells/hour. But O2 is only sparingly soluble in culture medium; the oxygen transfer rate (OTR) from gas phase into medium is about 17 μg /cm2
/hr.

Therefore, surface aeration can support about 50 x 106 cells in a 1 L culture vessel. When the medium depth in a culture is above 5 mm (especially, above 5 cm), aeration with a mixture of C02 and air becomes necessary to maintain adequate gas exchange.

Efficient aeration is achieved by bubbling air through the medium (sparging), but this may damage animal cells due to the high surface energy of the bubble and on the cell membrane. The damage can be reduced by using larger bubbles, lower gassing rates and by adding nonnutritional supplements like Pluronic F-6B (polyglycol), sodium carboxymethyl cellulose and polyvinylphyrrolidone (these protect cells from damage due to shear forces and bubbles, respectively). Silicone tubing (highly gas permeable) can be arranged inside the culture vessel (2-5 cm tubing of 30 m length for a 1000 L culture) and air is passed though the tube; however it is inconvenient to use.

Aeration may be achieved by medium perfusion, in which medium is continuously taken from culture vessel, passed through an oxygenation chamber and returned to the culture. The cells are removed from the medium taken for perfusion so that the medium can be suitably altered, e.g., for pH control. Perfusion is used with glass bead and, more particularly, with micro carrier systems.

Where considered safe and desirable, O2 supply in the culture vessel can be enhanced from the normal 21% to a higher value and the air pressure can be increased by 1 atmosphere. This increases the O2 solubility and diffusion rates in the medium, but there is a risk of O2 toxicity. The reactors used for large scale suspension cultures are of 3 main types:
(1) Stirred tank bioreactors, (2)
Continuous flow reactors, and
(3) Airlift fermenters.

Stirred Tank Bioreactors: These are glass (smaller vessels) or stainless steel (larger volumes) vessels of 1-1,000 1 or even 8,000 1 (Namalva cells grown for interferon; but in practice their maximum size is 20 1 since larger vessels are difficult to handle, autoclave and to agitate the culture effectively). These are closed systems with fixed volumes and

are usually agitated with motor-driven stirrers with considerable variation' in design details, e.g., water jacket in place of heater type temperature control, curved bottom for better mixing at low speeds, mirror internal finishes to reduce cell damage, etc. Many heteroploid cell lines can be grown in such vessels. The needs for research biochemical from cells are met from 2-50 1 reactors, while large scale reactors are mainly used for growing hybridoma cells for the production of monoclonal antibodies although their yields from cultured cells is only 1-2% of those obtained by passaging the cells through peritoneal cavity of mice.

Continuous-Flow Cultures: These culture systems are either of chemostat or turbidostat type. In both the types, cultures begin as a batch culture. In a chemostat type, inoculated cells grow to the maximum density when some nutrient, e.g., a vitamin, becomes growth limiting. Fresh medium is added after 24-48 hours of growth, at a constant rate (usually lower than the maximum growth rate of culture) and at an equal rate the culture is withdrawn.

When the rate of growth equals the rate of cell withdrawal, the cultures are in a 'steady state', and both the cell density and medium composition remain constant. One of the constituents of the medium is used at a lower concentration to make it growth-limiting. However, chemostat is the least efficient or controllable at the cell's maximum growth rate hence the steady-state growth rates in them are much lower than the maximum.

In contrast, in a turbidostat cells grow to achieve a predecided density (measured as turbidity using a photoelectric cell). At this point, a fixed volume of culture is withdrawn and the same volume of fresh normal (not having a growth-limiting factor) medium is added; this lowers the cell density or turbidity of the culture. Cells keep growing, and once the culture reaches the preset density the fixed volume of culture is replaced by fresh medium. This system works really well when the growth rate of the culture is close to the maximum for the cell line.
The continuous-flow cultures provide a continuous source of cells, and are suitable for product generation, e.g., for the production of viruses and interferons. It is often necessary to use a two- stage system in which the first stage supports cell growth, while the second stage promotes product generation.

Airlift Fermenters: Cultures in such vessels are both aerated and agitated by air (5% CO2 in air) bubbles introduced at the bottom of vessels . The vessel has an inner draft tube through which the air bubbles and the aerated medium rise since aerated medium is lighter than non- aerated one; this results in mixing of the culture as well as aeration. The air bubbles lift to the top of the medium and the air passes out through an outlet. The cells and the medium that lift out of the draft tube move down outside the tube and are recalculated. O2 supply is quite efficient but scaling up presents certain problems.

Fermenters of 2-90 L are commercially available, but 20,000 L fermenters are being used by biotechnology industries.

3. **Immobilized Cultures:**

Cultures based on immobilized cells offer the following several advantages:
(1) higher cell densities (50-200 x 10-6 cells/ml),
(2) stability and longevity of cultures,
(3) suitability for both suspension and monolayer cultures,
(4) protection of the cells from shear forces due to medium flow (in case of many systems), and
(5) less dependence of cells at higher densities on external supply of growth factors,which saves culture cost. There are the following two basic approaches to cell immobilization: (1) immurement (2) entrapment.

Immurement Cultures:
In such cultures, cells are confined within a medium permeable barrier. Hollow fibers packed in a cartridge are one such system. The medium is circulated through the fiber, while cells in suspension are present in the cartridge outside the fiber.

This is extremely effective for scales upto 1 1 and gives cell densities of 1-2 X 108 cells/ml; sophisticated units can yield upto 40 g monoclonal antibodies/month. Membranes permitting medium and gas diffusion are also used to develop bioreactors of this type; both small scale and large scale versions of membrane bioreactors are available commercially.

The cells may be encapsulated in a polymeric matrix by adsorption, covalent bonding, cross- linking or entrapment; the materials used as matrix are gelatin, polylysine, alginate and agarose. This approach (1) effectively protects cells from mechanical damage in large fermenters, and (2) allows production of hormones, antibodies, immunochemicals and enzymes over much longer periods than is possible in suspension cultures. (3) The medium diffuses freely into the matrix and into the cells, while cell products move out into the medium.

For production of larger molecules like monoclonal antibodies, agarose in a suspension of paraffin oil is preferable to alginate since the latter does not allow “diffusion of such products out of the alginate beads. Reactors of upto 3 1 are available commercially.

Entrapment Cultures:
In this approach, cells are held within an open matrix through which the medium flows freely. An example is the Opticell, in which the cells are entrapped within the porous ceramic walls of the unit.

Opticell units of upto 210 m2 surface area are available, which can yield upto 50 g monoclonal antibodies per day. The cells can also be enmeshed in cellulose fibres, e.g., DEAE, TLC, QAE, and TEAE. These fibers are autoclaved and washed as prescribed and added in a spinner/stirred bioreactor at a concentration of 3 g/1

Adherent Cell Culture	Suspension Cell Culture
Appropriate for most cell types, including primary cultures	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic)
Requires periodic passaging, but allows easy visual inspection under inverted microscope	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically	Does not require enzymatic or mechanical dissociation
Growth is limited by surface area, which may limit product yields	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Requires tissue-culture treated vessel	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exchange
Used for cytology, harvesting products continuously, and many research applications	Used for bulk protein production, batch harvesting, and many research applications

Table 2: Adherent Cell Culture vs Suspension Cell Culture

Hollow-fiber bioreactor (HFBR) systems:

HFBR systems are perfusion systems based on the principle of compartmentalization, where the cells are retained in a low-volume compartment at a very high concentration and medium is perfused through hollow fibers within the cell compartment. They have been used extensively to grow many types of mammalian cells under entrapped conditions, with the primary objective of obtaining large quantities of concentrated excreted proteins. HFBR systems offer a virtually shear-free environment for cell proliferation and simplicity of operation for the culture of cells to near-tissue-like densities.
HFBR systems have also been explored for the production of cells for tumor infiltrating lymphocytes (TIL) cell therapy, lymphokine activated killer (LAK) cell therapy and other cytotoxic T-cell therapies. Recently, expansion of human stem cells from fetal liver has also been reported. While these are excellent systems for producing high concentrations of excreted proteins, they suffer from several disadvantages when considering tissue engineering applications, in which the cells are the desired product. First, the culture environment is spatially inhomogeneous, and this creates potentially large concentration gradients of critical nutrients, as well as oxygen and pH.

Since hematopoietic differentiation is influenced by these culture variables, there would be less phenotypic control, leading to a loss of selectivity in the production of particular cell types. Second, when scaled-up, the space between fibers is not typically kept constant, which would significantly change the oxygen transfer characteristics of the system. Thus, while the culture performance of the HFBR systems might be adequate, the difficulties encountered in monitoring and controlling the culture environment suggest that a different type of reactor would be more appropriate for the expansion of primary hematopoietic cells.

CONTINUOUS FLOW CULTURES

Continuous culture is a set of techniques used to reproducibly cultivate microorganisms at submaximal growth rates at different growth limitations in such a way that the culture conditions remain virtually constant (in ‘steady state’) over extended periods of time. In the steady state, the growth of organisms can be studied in great detail under precisely controlled physiochemical states. Such conditions are amenable to a great deal of mathematical modelling that enables powerful quantitative analysis of microbial activities. Continuous culture principles first appeared in the literature near the middle of the twentieth century, notably from work performed in the labs of Herbert, Monod, and Novick. Since that time, continuous culture techniques have become common tools in both research and industry. A large diversity of continuous culture applications exists, of which only a modest subset will be mentioned in the present work. Focus will be on a number of classic and a few up-to-date examples of the use of continuous cultivation in various applications. As will be described, the use of continuous culture has enabled studies into several ecological phenomena, including the relationship between growth rate and intracellular metabolic fluxes, the transcriptional responses of microorganisms to various nutrient limitations, the competitive strategies between microorganisms at low nutrient concentrations, as well as the selection and competition between spontaneous or designed mutants for biotechnological applications. As synergistic tools continue becoming more powerful and widely available, the number of uses and the value of the classic continuous culture techniques will likely continue growing at a comparable rate.

APPARATUS

An open-type continuous-flow culture apparatus is described, with a working capacity of 500 ml culture. The glass culture vessel, of novel design, permits strict chemostat operation, and has the additional advantages of preventing wall growth, of operating without need for antifoam addition, and of growing filamentous microorganisms without plugging the apparatus. The agitation and mixing produce completely homogenous conditions in the culture; consequently representative sampling and product removal are obtained. Species of anaerobic and aerobic bacteria, yeasts, and micro- and macro-fungi have been grown in the apparatus, in runs which exceeded 4 months" duration. The apparatus is simple to use, reliable in operation, and suitable for wide laboratory use with non-pathogenic organisms.

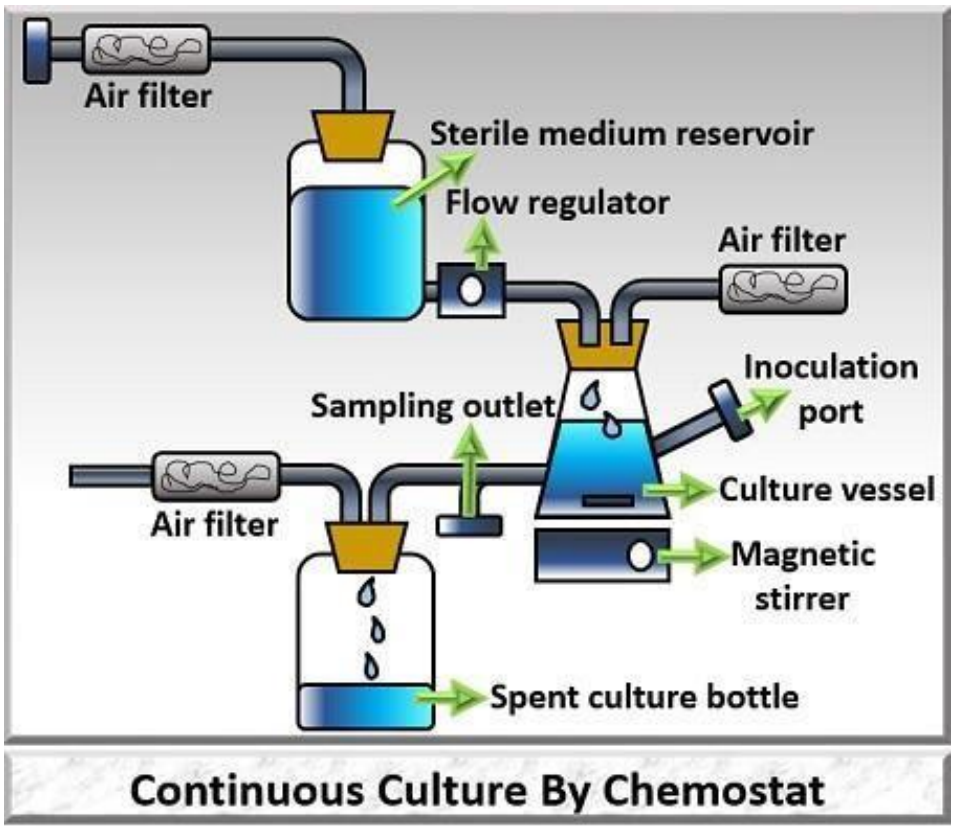


Figure 2.3: Continuous Culture By Chemostat

PREPARATION OF CONTINUOUS FLOW CULTURES

1. Continuous bioprocessing modes

The main aim of continuous cultivation is to maintain a continuous flow between the addition of
fresh media and the removal of cell suspension from the bioreactor simultaneously in order to maintain a constant reactor volume (Ozturk, 2006). In this way fresh nutrients are added, the product extracted, and possibly toxic by-products removed. All culture parameters remain constant, which demands additional control. It is also important to consider the danger of washing out the cells, which needs to decide for an appropriate dilution rate versus growth rate ratio.

Three types of continuous culture are described below:

Chemostat

Cell growth is limited and controlled by a nutrient addition (glucose, oxygen, glutamine and others) and depleted media with growth inhibitors is removed at approximately the same rate.

Turbidostat

A turbidostat dynamically adjusts the flow rate (and therefore the dilution rate) to keep the turbidity inside the vessel constant. The turbidity caused by cells in the culture medium can be tracked with sensors that measure light backscatter. Keeping the turbidity constant presents a new challenge for continuous operation. because cells need to be periodically removed independent of adding fresh medium separate from the harvesting process. Unlike chemostat operation, the cells need to be kept in the vessel for much of the process. When the culture density is too high, they must be removed, all while keeping a constant working volume.

Perfusion

This type of continuous bioprocessing mode is based on either retaining the cells in the bioreactor or recycling the cells back to the bioreactor. Here, fresh medium is provided and cell-free supernatant removed at the same rate (Ozturk, 2006). Various cell retention methods exist. Furthermore, in this mode the cell density increases constantly depending on the chosen media perfusion rate, also known as dilution rate factor D. The cell growth is limited to the nutrient or oxygen constraints or by waste product inhibition, leading to a quasi-steady-state of cells, metabolites and product concentration (Ozturk, 2006).

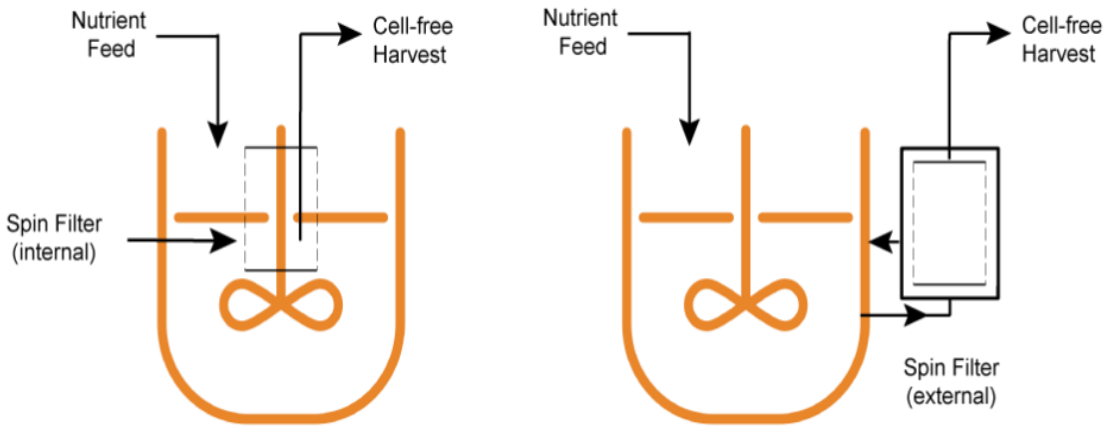


Figure 2.4: Cell Culture technology for pharmaceutical and cell-based therapies

2. Cell retention methods
Few cell retention methods are available. Here the most frequent ones are discussed:

Spin filter

This kind of cylindrical filter can be either attached internally to the drive shaft or externally to the bioreactor. The mesh size is small enough that the cells cannot pass through it to the inside. Depleted cell-free medium is removed by the dip tube inside the spin filter and fresh feed is added to balance the removal.

Alternating Tangential Flow (ATF)

The principle of ATF is based on alternately pumping the cell suspension to the filtration system
and then back to the bioreactor by using a fast diaphragm pump. For the separation of cells and product a standard hollow fiber membrane is applied [1].

Tangential Flow Filtration (TFF)

TFF is also known as cross-flow filtration, where the suspension is passed parallel to the filter system. Due to a pressure difference the filtered particles that are smaller than the filter pores pass through, whereas the larger components are retained and recycled back to the vessel.

Both versions of Tangential Flow Filtration are incredibly important because the technologies that they rely upon are low-shear and they do not damage the cells the way conventional peristaltic pumps may.

3. Setting up the antifoam pump for harvesting

Most bioreactors come with built-in peristaltic pumps, usually with one linked to antifoam. The antifoam pump typically adds liquid to the vessel from a reagent bottle and this happens when the foam probe is activated. In continuous bioprocesses, culture volume needs to be removed from the vessel into a specific harvest bottle. The easiest way to set this up is simply reverse the connections to the antifoam pump.

Once the vessel is autoclaved, the antifoam probe body should only ever be pulled upwards to support aseptic operation and the height of the probe should be adjusted with maximum stirring

and airflow set. Both agitation and gas flow can raise the height of the column of liquid in the vessel.

4. Configuring the feed pump

Feeding can only start when the culture has grown to a certain density, which means that a batch
phase of operation must be planned. Once this is over, feeding can be started manually or by timed events controlled by software. The feed rate from the media bottle into the vessel can be set manually or remotely via a script within the SCADA (Supervisory Control And Data Acquisition) software.

The simple rules for feeding are:

- Most continuous cultures run as chemostats. Addition of only one substance limits growth in the culture. The maximum feed rate will depend on the growth of the specific microbe on a defined growth-limiting substrate. It is important to take care that the cells are not washed out, which means the liquid exchange should be slower.

- The media exchange is defined by the dilution rate (D), usually in units per hour (h^{-1}). It describes the relationship between the flow of medium into the bioreactor (F), that can be expressed in $L \cdot h^{-1}$, and culture volume within the bioreactor (V) in L

$$D = F / V$$

- To reach a culture at steady state, the Dilution rate (D) must be equal the specific growth (μ) thereby keeping a constant biomass concentration inside the bioreactor.

$$\mu = D$$

5. Configuring the harvest pump

The antifoam pump is now a harvest pump and needs to be set up as such:

- The take-off (harvest) must be set to a faster rate than the inlet feed. This makes sure the vessel cannot overfill.
- As the antifoam pump works on a shot and delay basis, this usually means running a longer ON time and a shorter OFF time than usual (dose and delay).
- The direction of flow is from a dip tube in the vessel through the antifoam pump and into a harvest bottle.

Both feed and harvest bottles need to be larger than those used for reagents. Use the working volume of the vessel and the number of exchanges per day to work out the volume of feed consumed in 24 hours. The capacity of both bottles must be larger than this. The feed bottle needs a dip tube and gas filter connection. The harvest bottle needs only a short inlet pipe and filter connection. It is common to place the feed bottle above the vessel and the harvest bottle below. Gravity can then aid the liquid flow.

6. Making aseptic connections

As cultures can last for weeks or months, there is no way to only use a single feed and harvest bottle. If they were large enough, they would be too heavy to move easily, once filled. So, exchange of feed and harvest bottles is necessary.

Luer connectors make this task secure and rapid. The new bottle is fitted with one part of a Luer connector, filled and autoclaved. The open end of the connector can be covered with aluminium foil to reduce the risk of contamination. When exchange is needed, the existing Luer connector is

sprayed with alcohol and opened. The new connector is uncovered and rapidly twisted onto the existing part. With a little care this operation can be securely repeated many times for microbial processes. For cell culture applications working with a tube welder ensures a sterile exchange of the bottles. The tubing can then be quickly primed.

Summary:

The theory of continuous culture is well understood. The practical details to make a success of it are not so easy to find. Some of the key elements have been mentioned to help you i.e.

- How to simply change the use of an antifoam pump to make it run as a harvest pump.
- Configuration of the feed and harvest elements of a continuous process.
- Dealing with the need for many aseptic connections during the lifetime of the process.
- Condensing the key information into a simple workflow for a chemostat.

The best way to start with continuous culture is to look out for bioreactors where this is a standard option. Everything you need to get started should be available, along with clear information about its use.

Immobilization Cell Culture

Types of Cell culture

- **Adherent Cell Culture:** When the primary cells are seeded on or adhered to the culture medium in high density.
- **Suspension Cell Culture:** consists of single cells, small cell groups and larger cell aggregates dispersed in a liquid medium and actively grow.
- **Immobilization Cell Culture:** the cells are either encapsulate or adsorbed or entrapped within a polymeric or open matrix.

Figure 2.5: Suspension Culture and Immobilization Culture

Immobilization is often confused with mineralisation

- Immobilization is closely related to mineralisation as both are due to inorganic organic compounds.
- Any media has inorganic as well as organic compounds in its composition.
- Now when a cell consume inorganic compounds for its metabolism and other organic compounds needed to be grown then they are said to be "immobilising", while when a cell excrete inorganic waste compounds then they are said to be "mineralising".

Types of cell immobilisation

We have already heard about enzyme immobilisation in Enzymology, Enzyme can be immobilized through the following techniques.

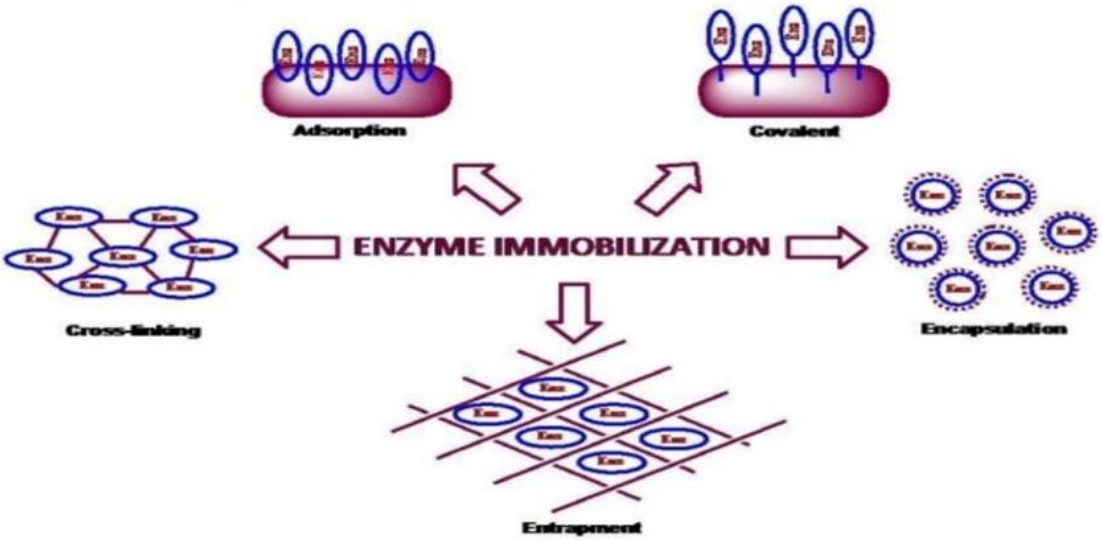


Figure 2.6: Enzyme Immobilization

Similarly Cells can be immobilized in cell culture.
The cells can be immobilised by

- covalently attached to the base Adsorbed or absorbed
- Cross-linked
- Encapsulate
- Assembled to a protein
- Entrapped

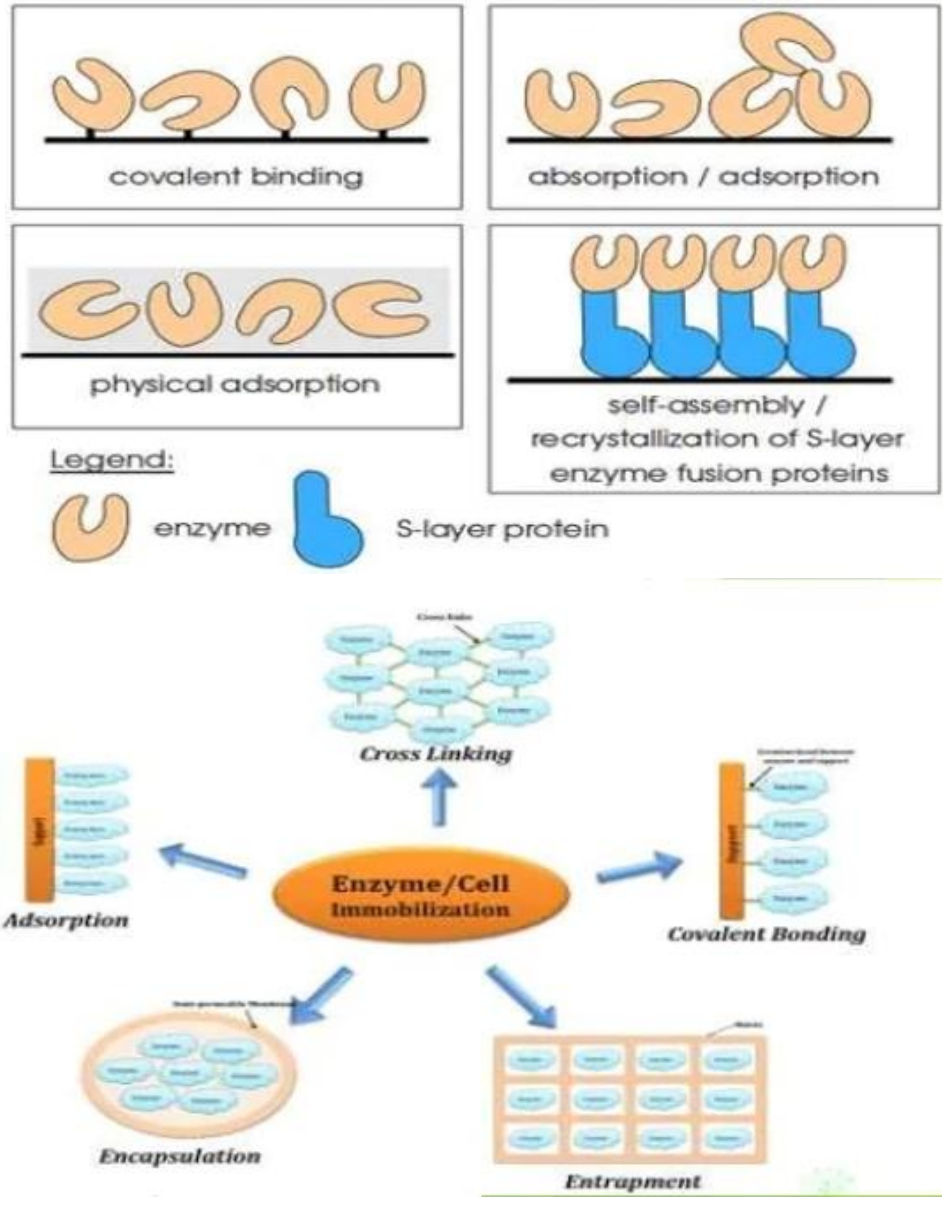


Figure 2.7: Enzyme Immobilization

Different types of cell immobilization

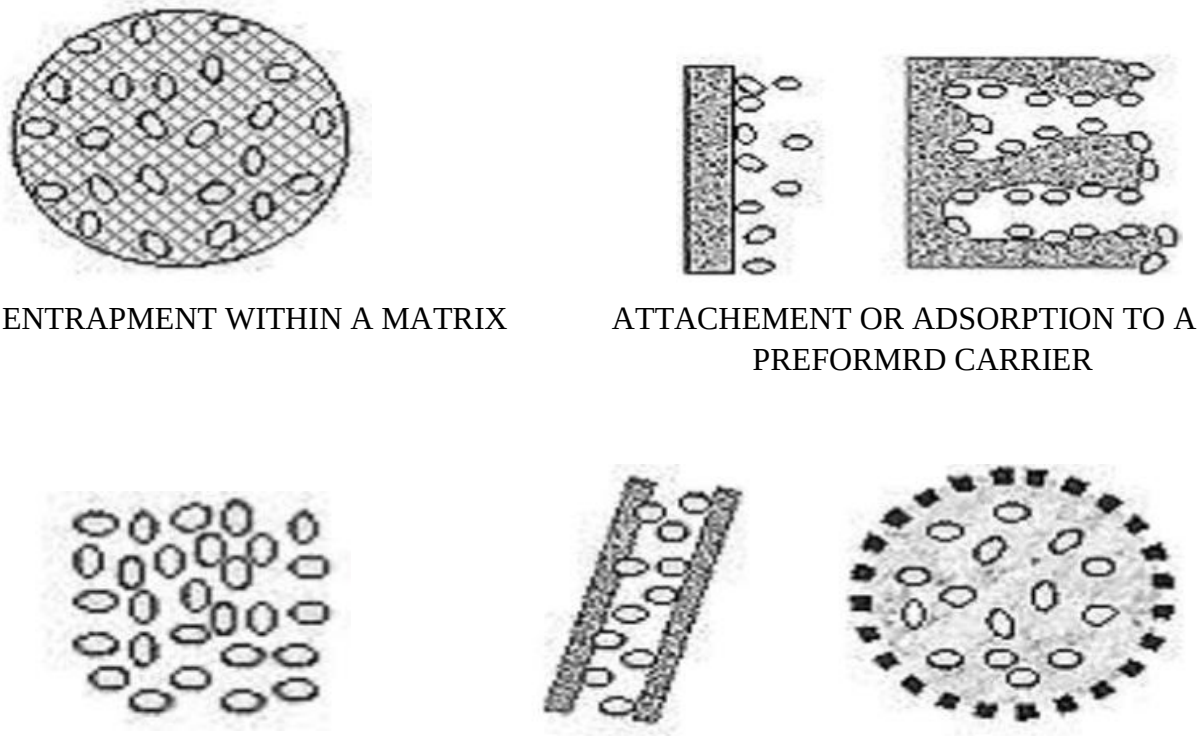
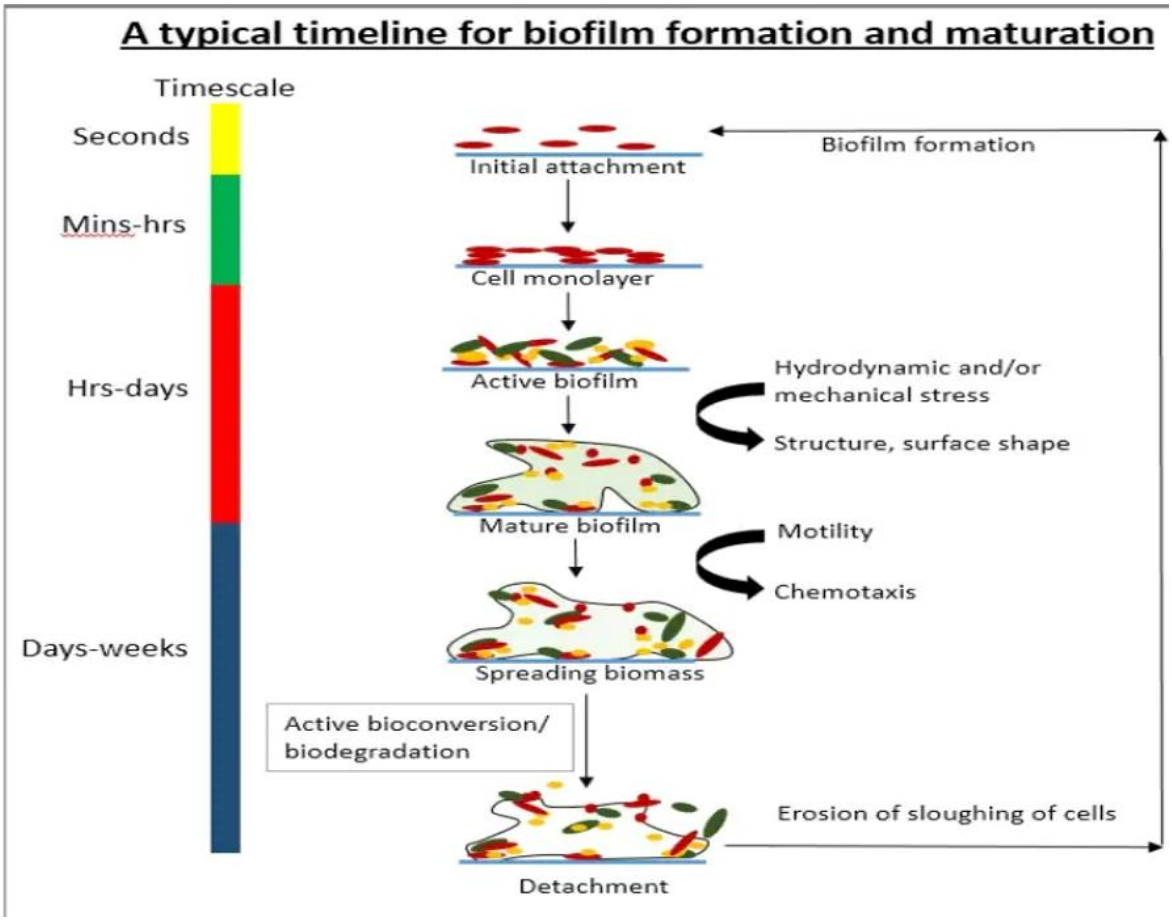


Figure 2.8: Types of Cell Immobilization



Physical Adsorption

Figure 2.9: Timeline for Biofilm formation and Maturation

The two basic cell immobilization techniques include

- ✓ immurement cinturel Encapstation
- ✓ Entrapment

Immurement Culture/Encapsulation

- ✓ Cells are encapsulated in a polymeric matrix by adsorption.
- ✓ Materials used in matrix: gelatin, polylysine, alginate(most common), Agarose. Medium diffuses freely into the matrix and into the cells, while product moves

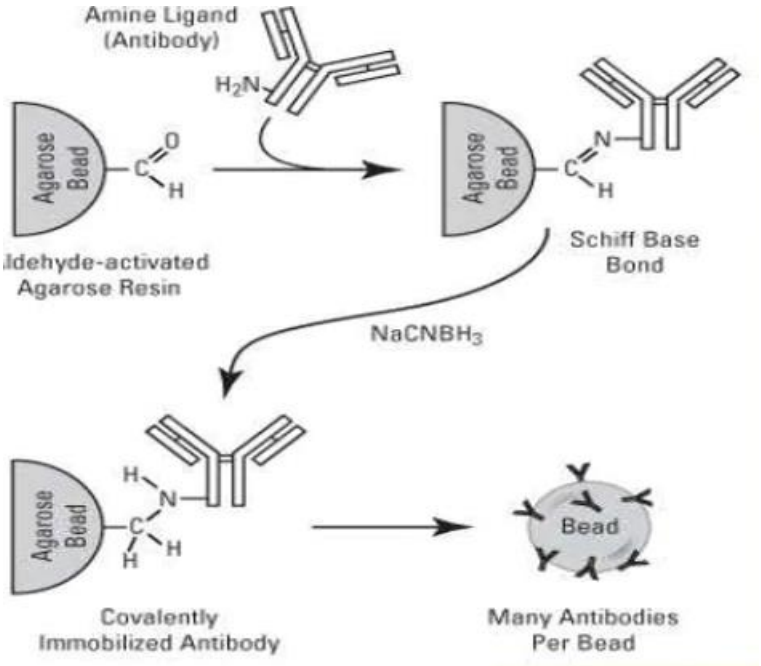
- ✓ out into the medium. For larger molecule production like MAB, Agarose in suspension of paraffin oil is preferable to alginate since alginate does not allow diffusion of products out of alginate beads.

Uses

- Encapsulation protects cells from mechanical damage in large fermenters. Production of hormones, antibodies, immunochemical and enzymes aver much longer periods than possible in suspension culture.

- Helps in MAB production.

- Immobilization of Antibodies



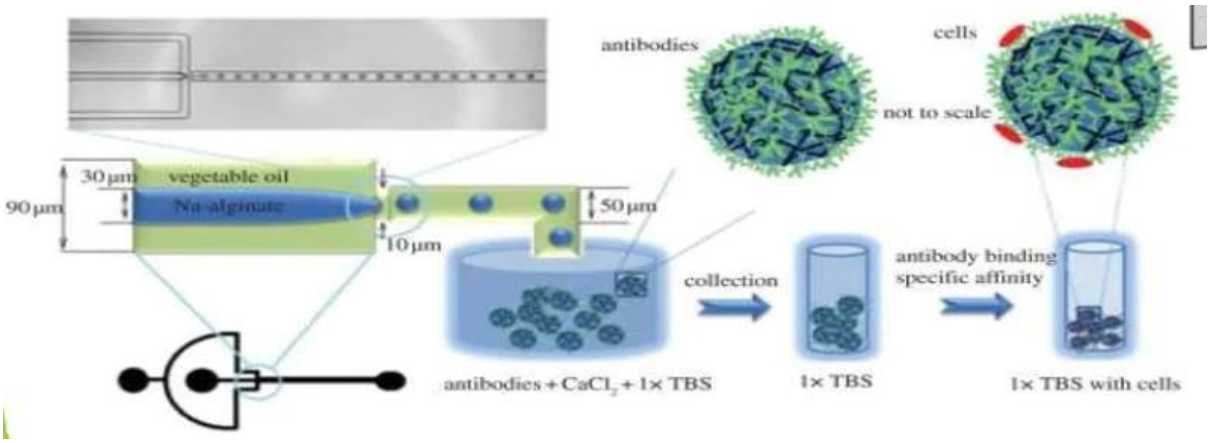


Figure 2.10: Immobilization of Antibodies

Entrapment culture

- Cells are held within an open matrix through which the medium flows freely.
- Cells may be entrapped within porous ceramic walls of the entrapping unit.

Use:

- Porous micro carriers (170µm-6000µm) made up of gelatin or collagen or glass or cellulose are arranged as fixed bed or fluidized bed reactors or used in stirred bioreactors.

Cells can also be enmeshed in cellulose fibres.

Use:

- These fibres are autoclaved and washed and added in stirred bioreactors at a certain conc. (3g/L).

- This yields upto 50g MAB/day.

Few of Immobilized Cells

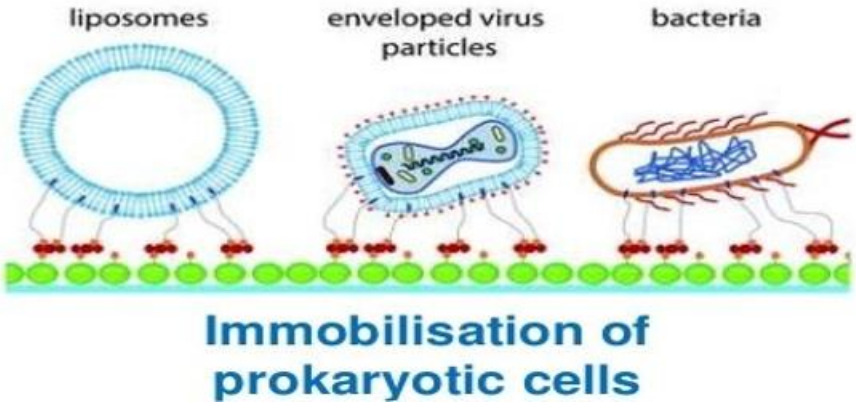


Figure 2.11: Immobilisation of prokaryotic cells

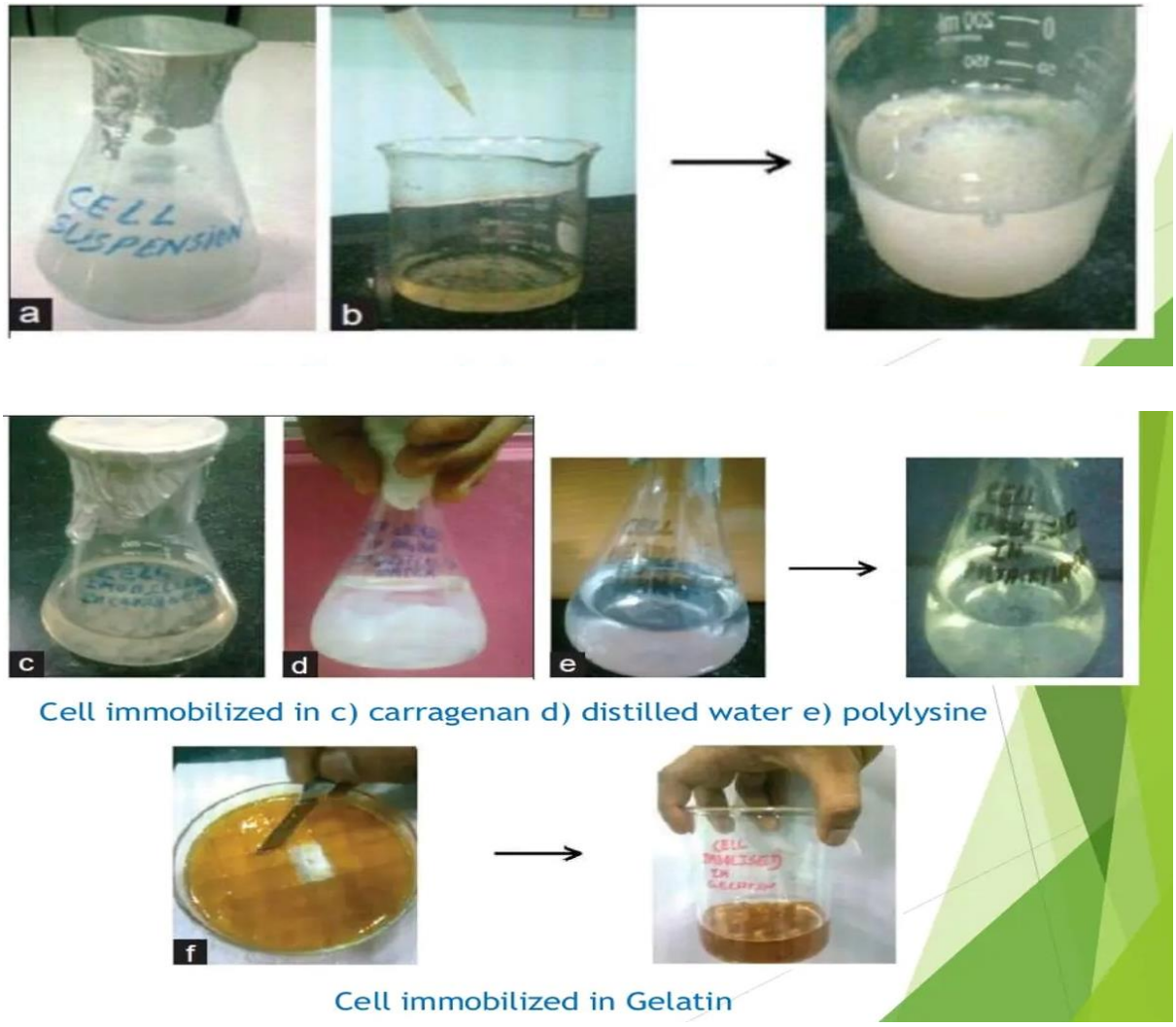


Figure 2.12: Cell Immobilized in Ca-Alginate

Uses of immobilized cell culture

- Help in making bio-transformants, biosensors.
- Secondary metabolite production.
- Biological washing powders.
- Food Industry.
- Bioreactors.
- Experimental waste water treatment.

Figure 2.13: Immobilisation of microalgae

Figure 2.14: Immobilized algae used in bioreactor

Ca-Alginate Immobilization of Yeast cells

Immobilized Cell Preparation:

- Sodium alginate in growth medium.
- Stir until all sodium alginate is completely dissolved. No clump formation.
- Suspend wet cells in the alginate solution prepared in the above step. Let air bubbles escape. Drip the yeast alginate mixture. The calcium crosslinking solution is agitated on a magnetic stirrer.
- Gel formation can be achieved at room temperature as soon as the sodium alginate drops come in direct contact with the calcium solution.

- Relatively small alginate beads are preferred to minimize the mass transfer resistance. A diameter of 0.52mm can be readily achieved with a syringe and a needle. The beads should fully harden in 12hours.

- Wash the beads with a fresh calcium crosslinking solution.

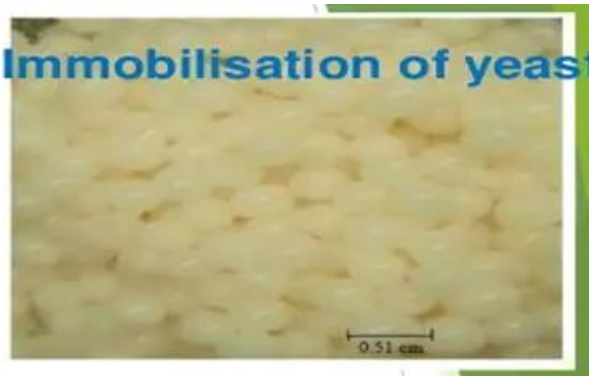


Figure 2.15: Immobilisation of yeast

Ca-Alginate Immobilization of Yeast cells

Immobilized Cell Reactor Construction:

- Construct an immobilized cell reactor with a 500ml Erlenmeyer flask fitted.

- Place the hardened beads in the flask and seal it with a rubber stopper with appropriate hose connections:

- Make all necessary connections. Start the experiment by filling the flask with the growth. media (100g/1 glucose) to the working volume of 350ml.

Immobilized Cell Reactor:

- Then following sequence of events will be monitored both online and offline.
- The responsibilities of online data acquisition and offline sample collection and analysis will be shared by the entire class; the exact assignment will be determined in class A microcomputer will be programmed to take data on the glucose concentration and the rate of NH OH addition needed to maintain the pH at 4.0. The offline samples will be analyzed for the optical density (for free cell concentration), glucose concentration, and ethanol concentration.

- Furthermore, the liquid and gas flow rate will be measured with a graduatod cylinder.

- The reactor will be operated in a batch manner until no more glucose is utilized. This can be detected with the leveling off in the glucose concentration.

- The reactor will be operated in a batch manner until no more glucose is utilized. This can be detected with the leveling off in the glucose concentration.

- Furthermore, the liquid and gas flow rate will be measured with a graduated cylinder.

- The reactor will be operated in a batch manner until no more glucose is utilized. This can be detected with the leveling off in the glucose concentration.

- Substrate feeding will then commence at the rate of 0.4/hr. Record the substrate flow rate. The approach to the first steady state during the startup will be followed.

- Various parameters (nitrogen consumption rate, carbon dioxide evolution rate, glucose concentration, ethanol concentration, and free cell level) at the high steady state recorded.

- Decrease the substrate feeding rate to 0.2 /hr., Measure the substrate flow rate and are follow the transient approach to the new low steady state.

- Repeat above parts for the new steady state.

- If time permits, continue shifting the flow rate and obtain more information on steady states Continue operating the bioreactor until noticeable deterioration in the performance is detected due to gel swelling, cell death, or severe contamination.

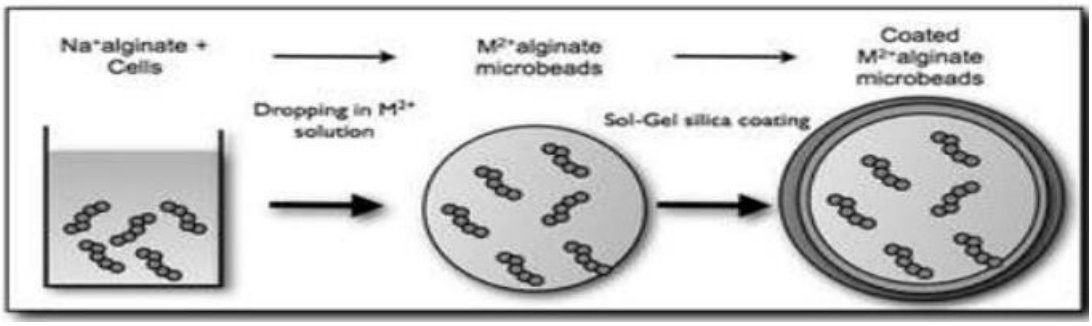


Figure 2.16: Sol-Gel silica coating

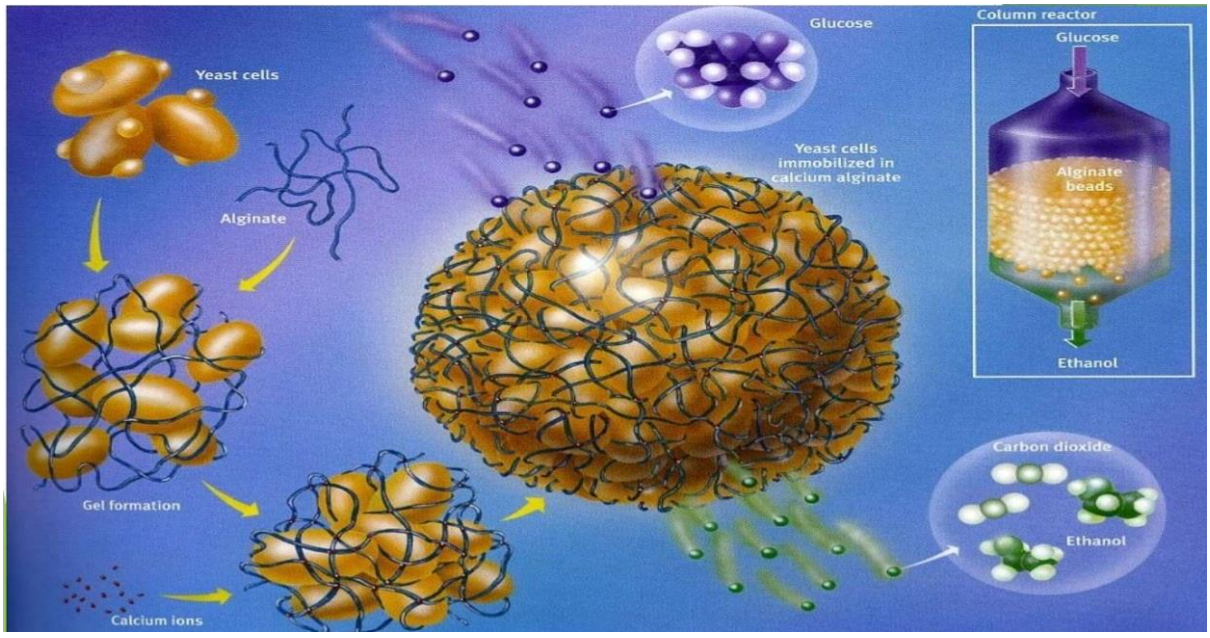


Figure 2.17: Ca-Algínate Immobilization of Yeast cells and large scale production of ethanol

Bioreactors immobilised and cells

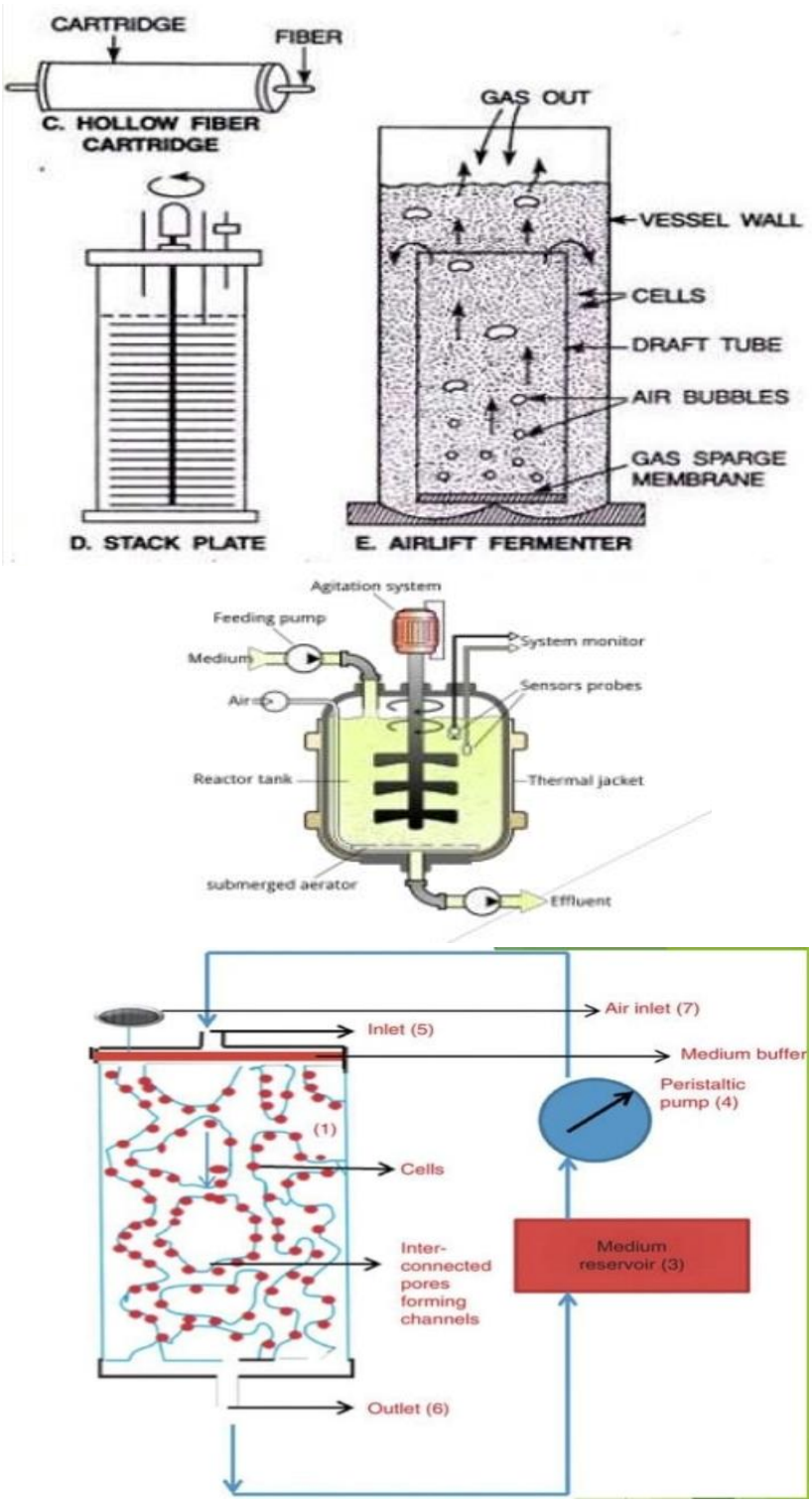
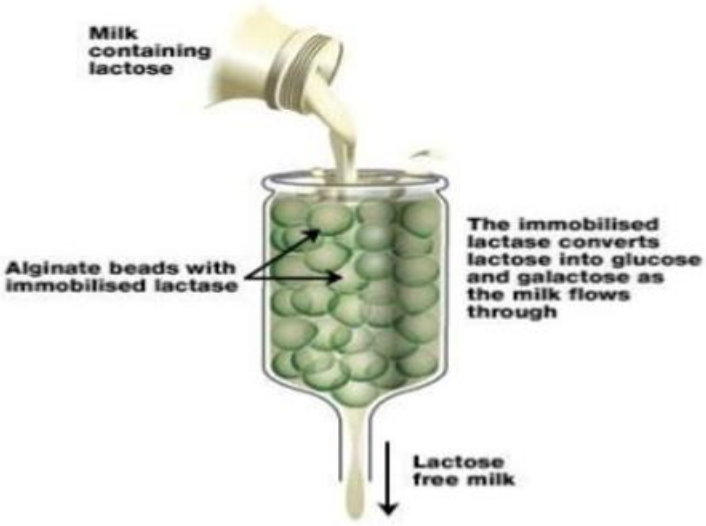


Figure 2.18: Types of Bioreactors



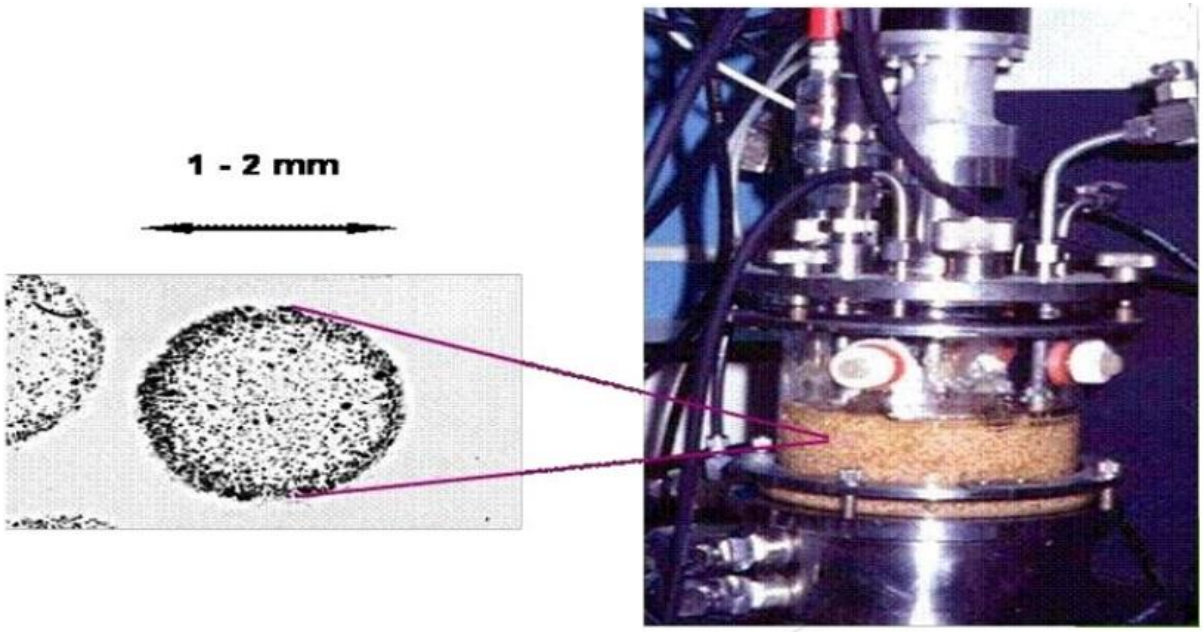


Figure 2.19: Immobilized cells and their use in Bioreactors

Use of cell Immobilization

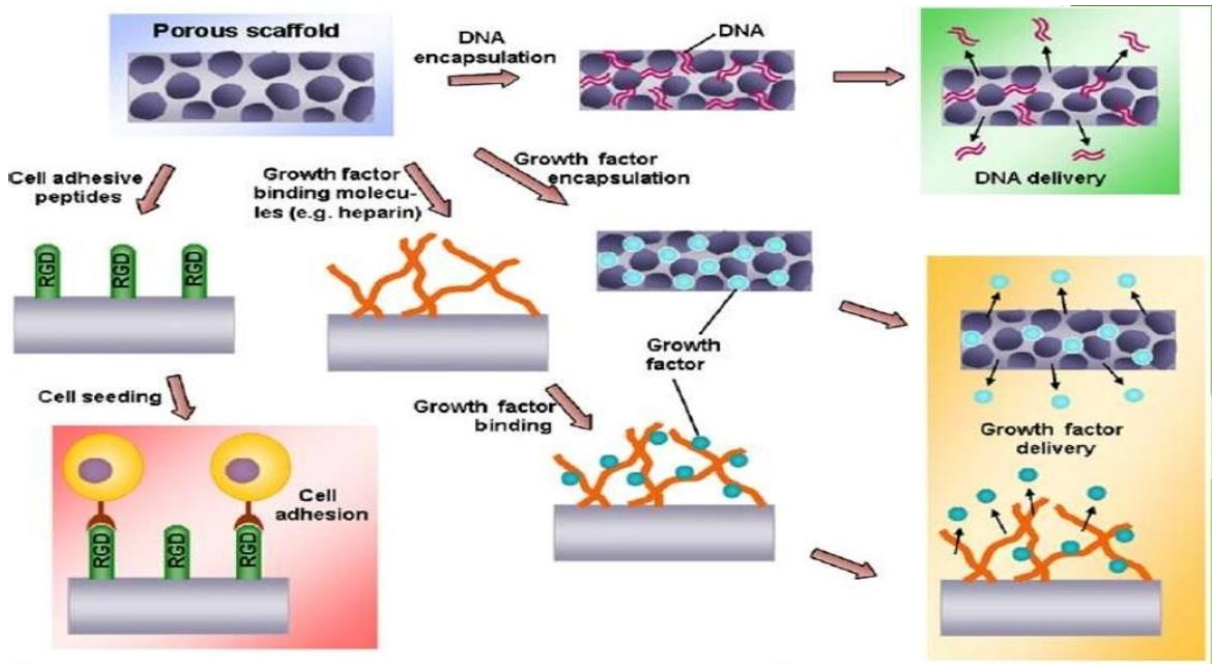


Figure 2.20: Cell Immobilization

Other benefits of using Immobilized cell culture

- Gives higher cell density.
- Stability and longevity of cultures.
- Can be applicable to both suspension or monolayer culture(adherent).
- Many systems protect the cells from shear forces due to medium flow.
- Less dependence of cells at higher densities on external supply of growth factors which saves culture cost.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 3 – TRANSGENIC ANIMALS – SBTA 1601

UNIT-3 NOTES

Concepts of Transgenic Animal Technology

- Transgenic animals are animals that have had a foreign gene deliberately inserted into their genome.
- The foreign gene is constructed using recombinant DNA methodology.
- Here is the picture of two transgenic mice that are genetically modified by having a green fluorescent protein and it glows under blue light.

Timeline and key events

- In 1929, Jackson memorial laboratories developed mice to study cancer and other diseases
- In September 1980, the first mice were created by recombinant DNA technology.
- In November 1980, Capecchi published a technique to inject DNA directly into the nuclei of cultured mammalian cells by using glass micropipettes.
- On July 5, 1996, the first cloned mammal (Dolly, the sheep) was born and the work was done by Wilmut. • In September 2006, the first human monoclonal antibody-drug was approved.
- On September 23, 2015, Beijing Genomic Institute created micropigs with the help of the TALENS gene editing technique
- On October 5, 2015, US Scientists from Harvard University modified 60 genes in pig embryos to create pig organs that are suitable for humans.

Advantages of Transgenic Animals:

- Gene requires certain cellular mechanisms to help for the production of protein. The animals used for transgenic purpose naturally carry the mechanism needed to produce complex protein. Theses mechanism is absent in cell culture.
- Expression through cell culture or bacterial culture requires constant monitoring and sampling.
- The isolation and purification of expressed protein in the conventional method are more difficult than purifying proteins from an animal's milk or body fluid.
- It is more cost-effective as the product is efficiently passed through milk with an average yield of 53% and with 99% purity.
- It has been estimated that transgenic animal can produce in its lifetime $100 to $200 million worth of pharmaceuticals.

Disadvantages of Transgenic Animals:

- Transgenic animal project is extremely expensive.
- The generation of transgenic animals is also expensive, because of long gestation period, litter size and higher maintenance cost of the recipient animals.

- There may be high mortality rate and other deleterious effects on animals used by researchers to create transgenic breeds. It has been observed that transgenic pigs having enhanced growth rate and efficient feed conversion exhibit reduced reproductive performance and may suffer from arthritis and dermatitis etc.
- Similarly, transgenic sheep expressing growth horm6ne may show diabetic like conditions. Mayer argues that we do not understand the long term effects of genetic engineering on animals.
- A large number of recipients is required for embryo transfer because of low transgenesis rate.
- Transgenic foods have been produced and offer better productivity in terms of both yield and quantity. However, there are some apprehension about the safety of transgenic foods.

Transgenic Techniques

A variety of methods can be used to introduce new genetic material into animals, and new methods continue to be developed and refined. Here is a brief overview of some techniques:

■ **Retrovirus-mediated transgenics** is accomplished by infecting mouse embryos with retroviruses
before the embryos are implanted. The retrovirus acts as a vector for the new DNA. This method is restricted in its applications because the size of the **transgene** (or *trans*ferred *gene*tic material) is limited and the virus's own genetic sequence can interfere with the process, making transgenics a rather hit-and-miss project

Chimeric Animals Generation and Cloning Cloning

Clone: a collection of molecules or cells, all identical to an original molecule or cell

- To "clone, a gene" is to make many copies of it-for example, in a population of bacteria
- Gene can be an exact copy of a natural gene
- Gene can be an altered version of a natural gene
- Recombinant DNA technology makes it possible

Plasmids

Naturally occurring extrachromosomal DNA

- Plasmids are circular dsDNA
- Plasmids can be cleaved by restriction enzymes, leaving sticky ends
- Artificial plasmids can be constructed by linking new DNA fragments to the sticky ends of plasmid

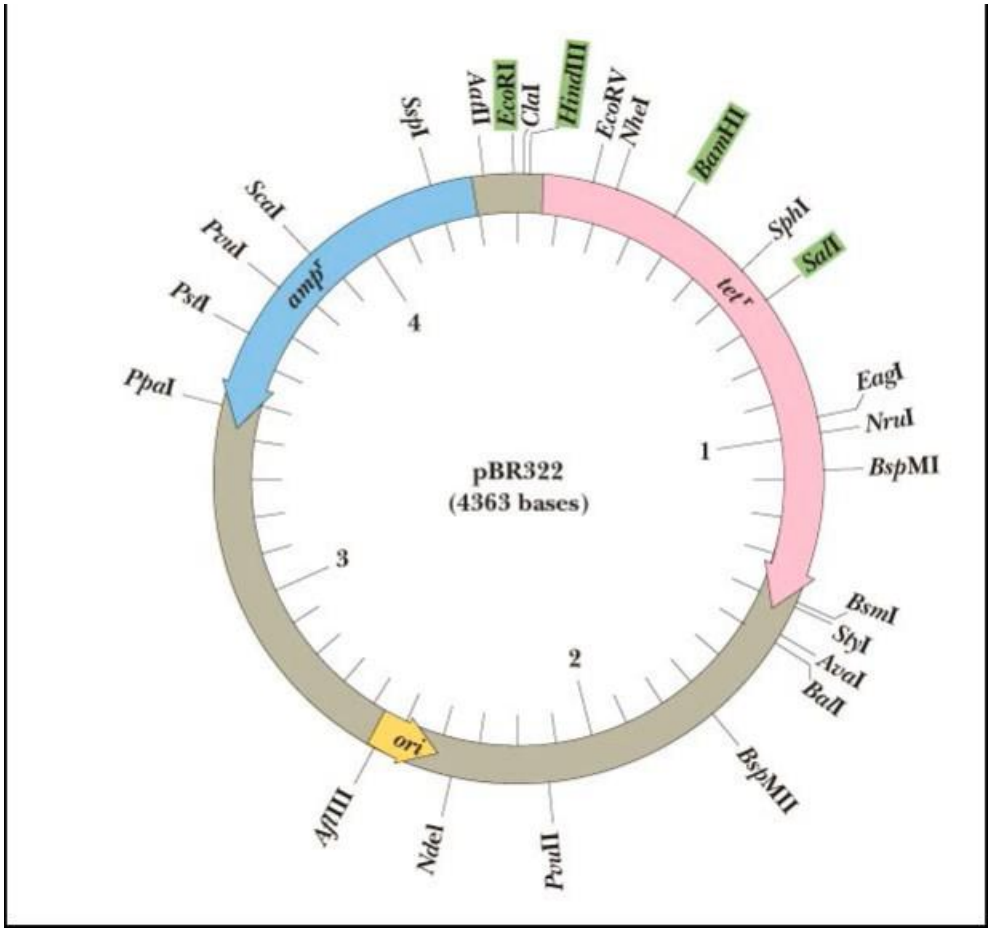

Figure 3: pBR322 plasmid

Cloning Vectors

Plasmids that can be modified to carry new genes

Plasmids useful as cloning vectors must have

- a replicator (origin of replication)
- a selectable marker (antibiotic resistance gene)
- a cloning site (site where insertion of foreign DNA will not disrupt replication or inactivate essential markers

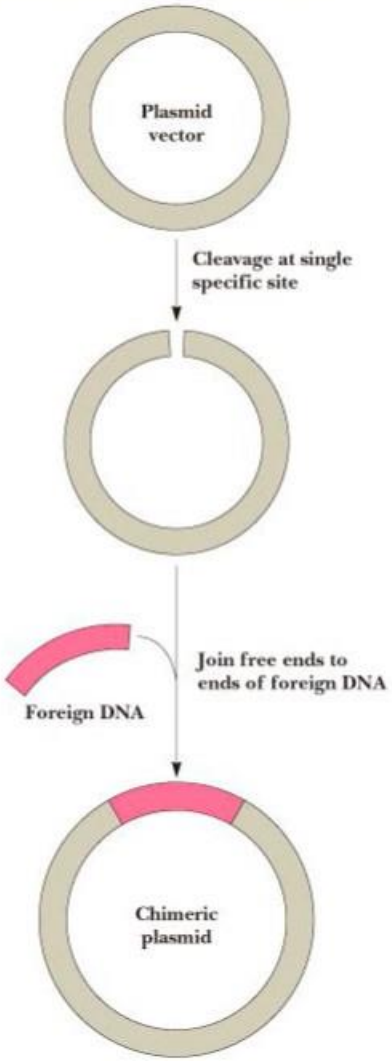

Figure 3.1: Production of recombinant DNA

Chimeric Plasmids

Named for mythological beasts with body parts from several creatures

- After cleavage of a plasmid with a restriction enzyme, a foreign DNA fragment can be inserted
- Ends of the plasmid/fragment are closed to form a "recombinant plasmid"
- A plasmid can replicate when placed in a suitable bacterial host

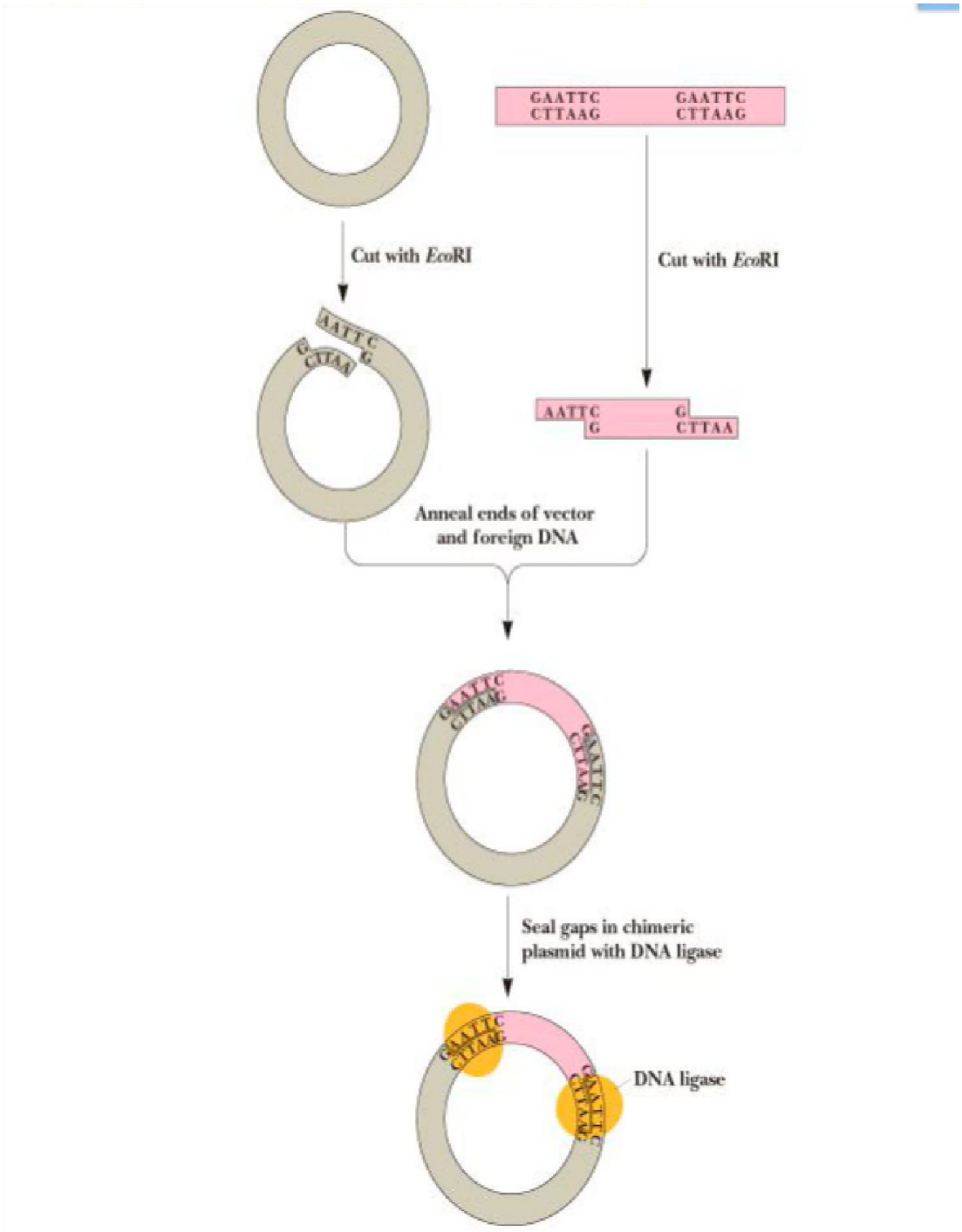

Figure 3.2: Recombinant DNA

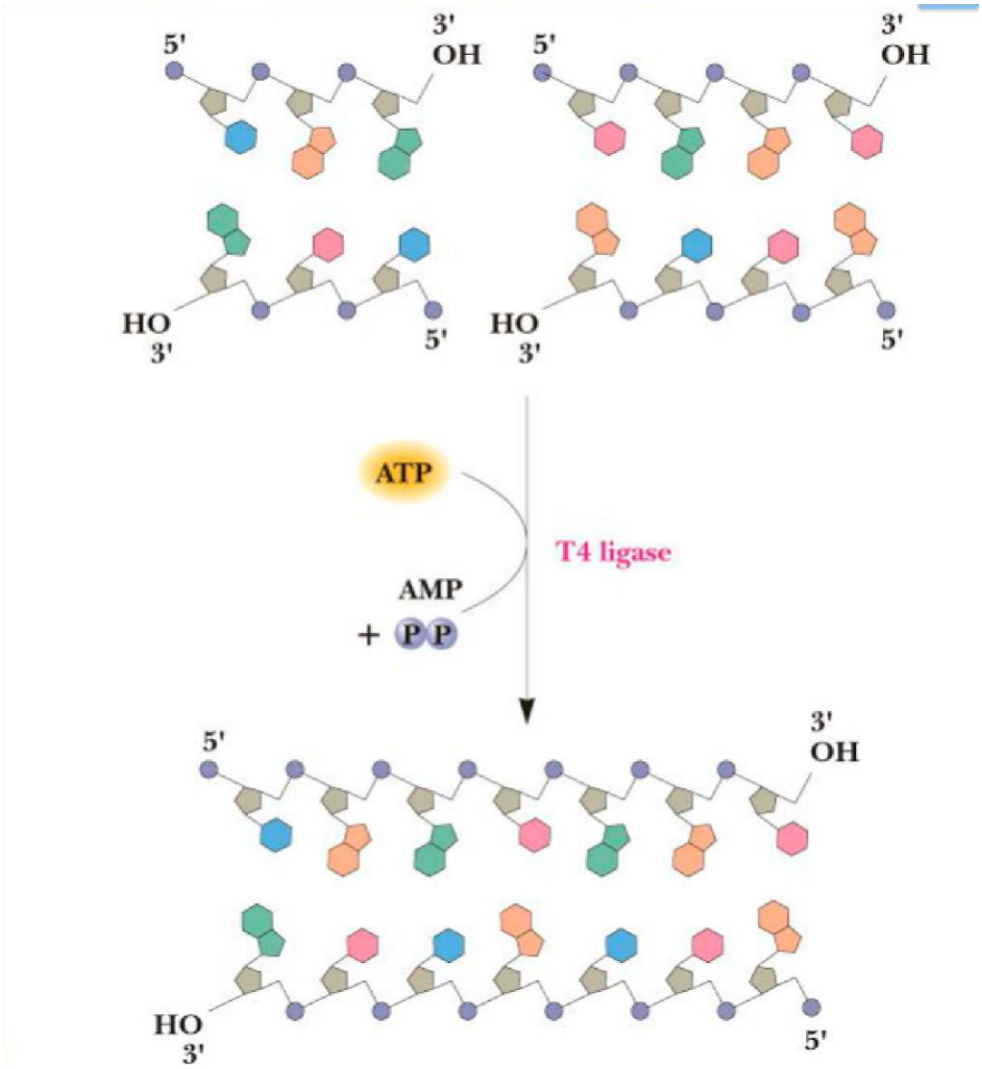

Figure 3.3: T4 DNA Ligase

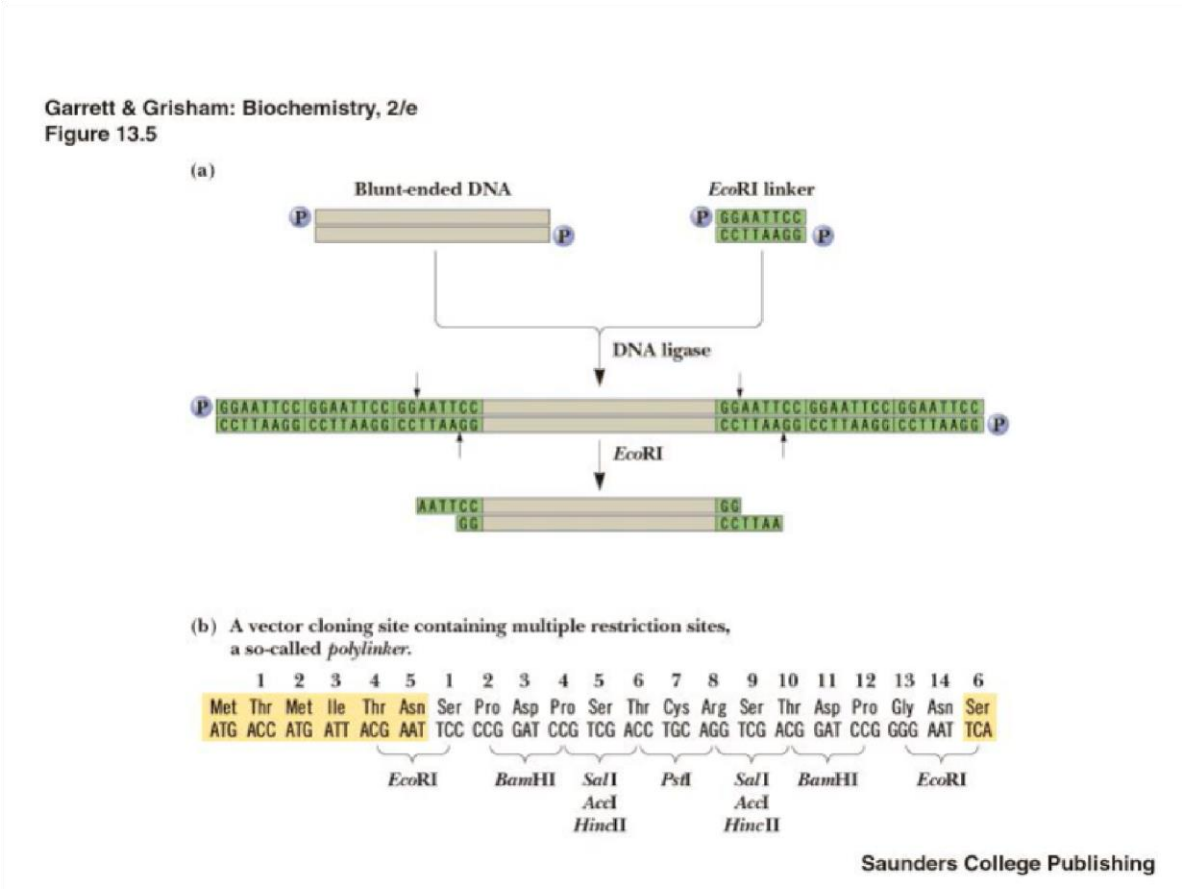

Figure 3.4: (a) The use of linkers to create tailor-made ends on cloning fragments. Synthetic oligonucleotide duplexes whose sequences represent EcoRI restriction sites are blunt-end ligated to a DNA molecule using T4 DNA ligase. Note that the ligation reaction can add multiple linkers on each end of the blunt-ended DNA. EcoRI digestion removes all but the terminal one, leaving the desired 5'-overhangs. (b) Cloning vectors often have polylinkers consisting of a multiple array of restriction sites at their cloning sites, so restriction fragments generated by a variety of endonucleases can be incorporated into the vector. Note that the polylinker is engineered not only to have multiple restriction sites but also to have an uninterrupted sequence of codons, so this region of the vector has the potential for translation into protein. The sequence shown is the cloning site for the vectors M13mp7 and pUC7; the colored amino acid residues are contiguous with the coding sequence of the lacZ gene carried by this vector

Directional Cloning

Often one desires to insert foreign DNA in a particular orientation

- This can be done by making two cleavages with two different restriction enzymes
- Construct foreign DNA with the same two restriction enzymes
- Foreign DNA can only be inserted in one direction

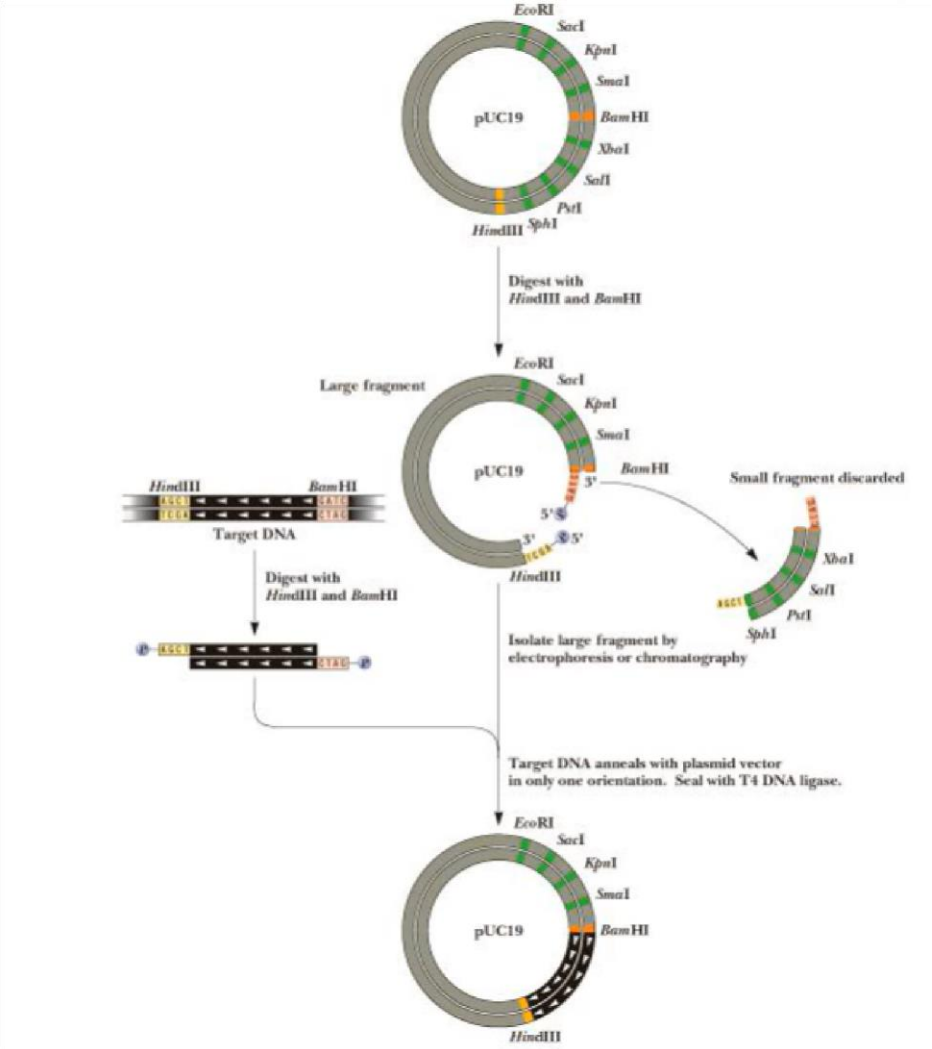

Figure 3.5: Directional Cloning

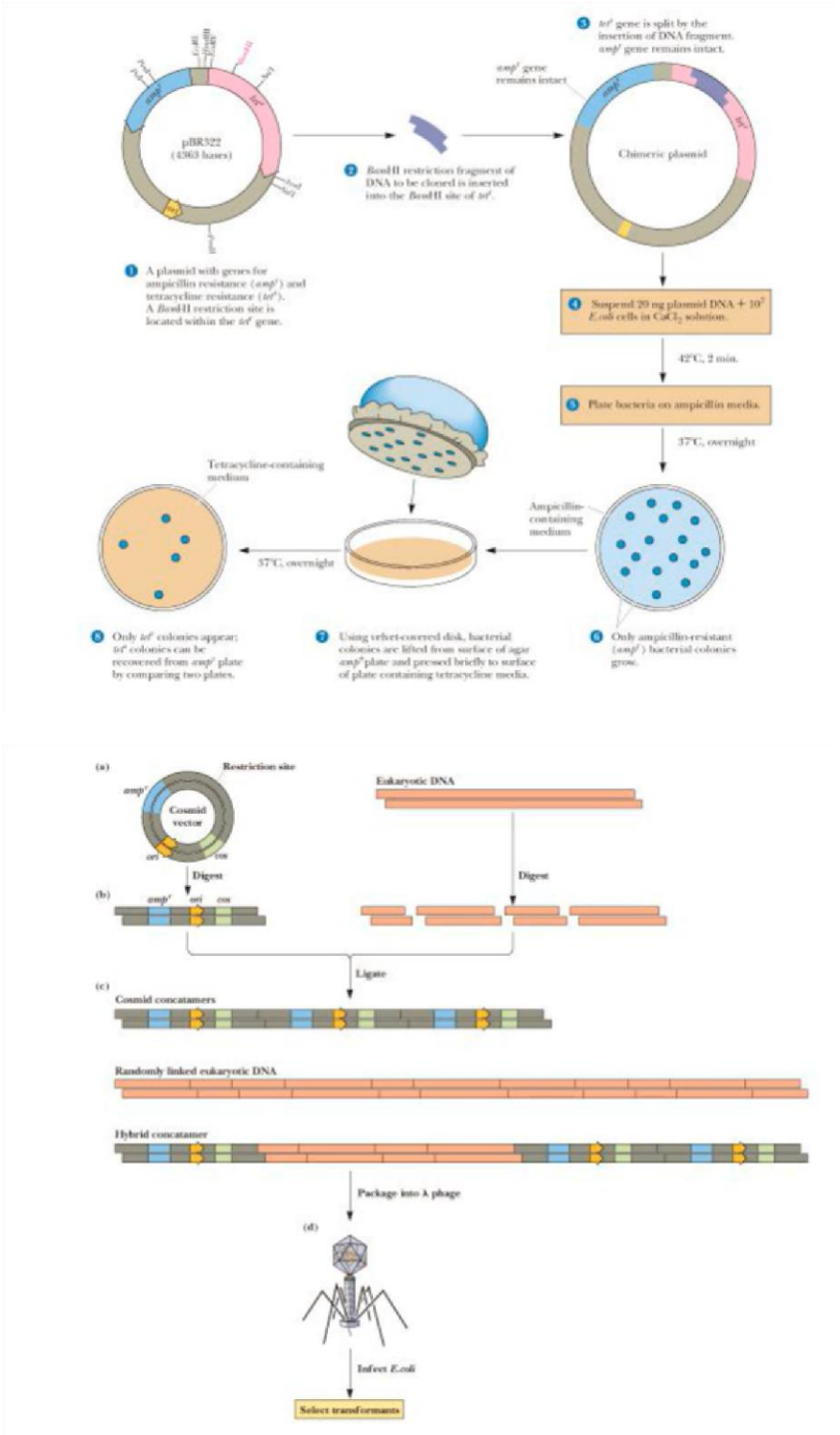

Figure 3.6: Selection of Transformed Cells

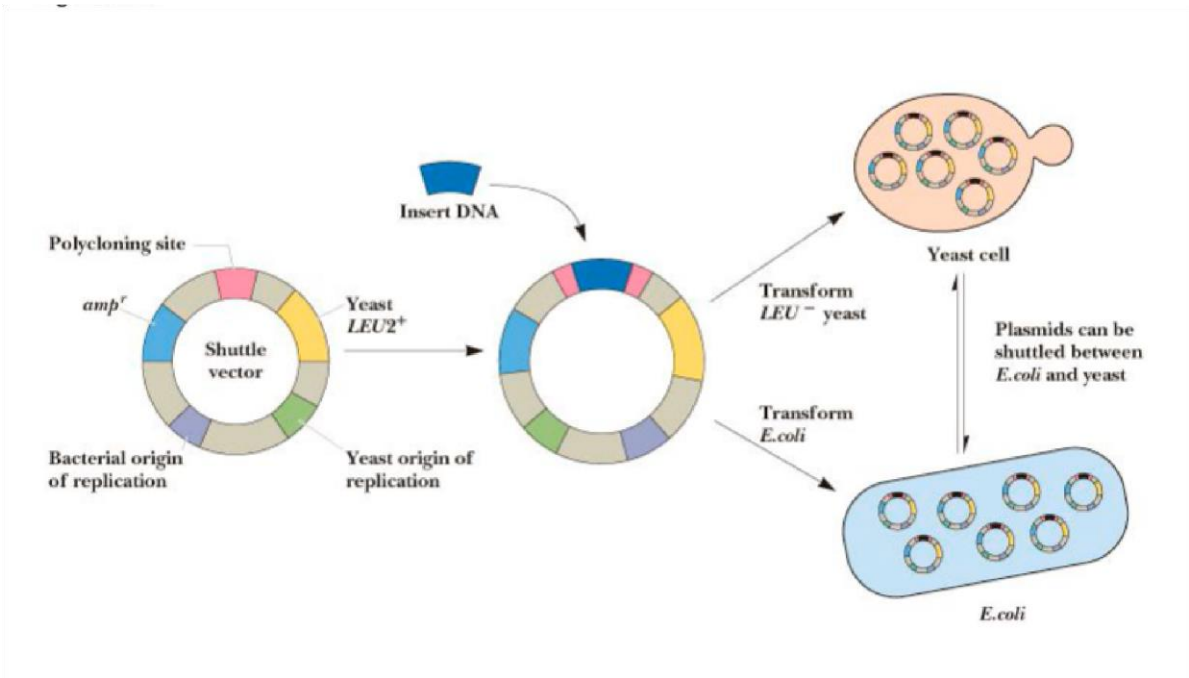

Figure 3.7: Shuttle Vector

DNA Libraries

Sets of cloned DNA fragments that together represent the genes of a particular organism

- Any particular gene may represent a tiny, tiny fraction of the DNA in a given cell
- Can't isolate it directly
- The trick is to find the fragment or fragments in the library that contains the desired gene

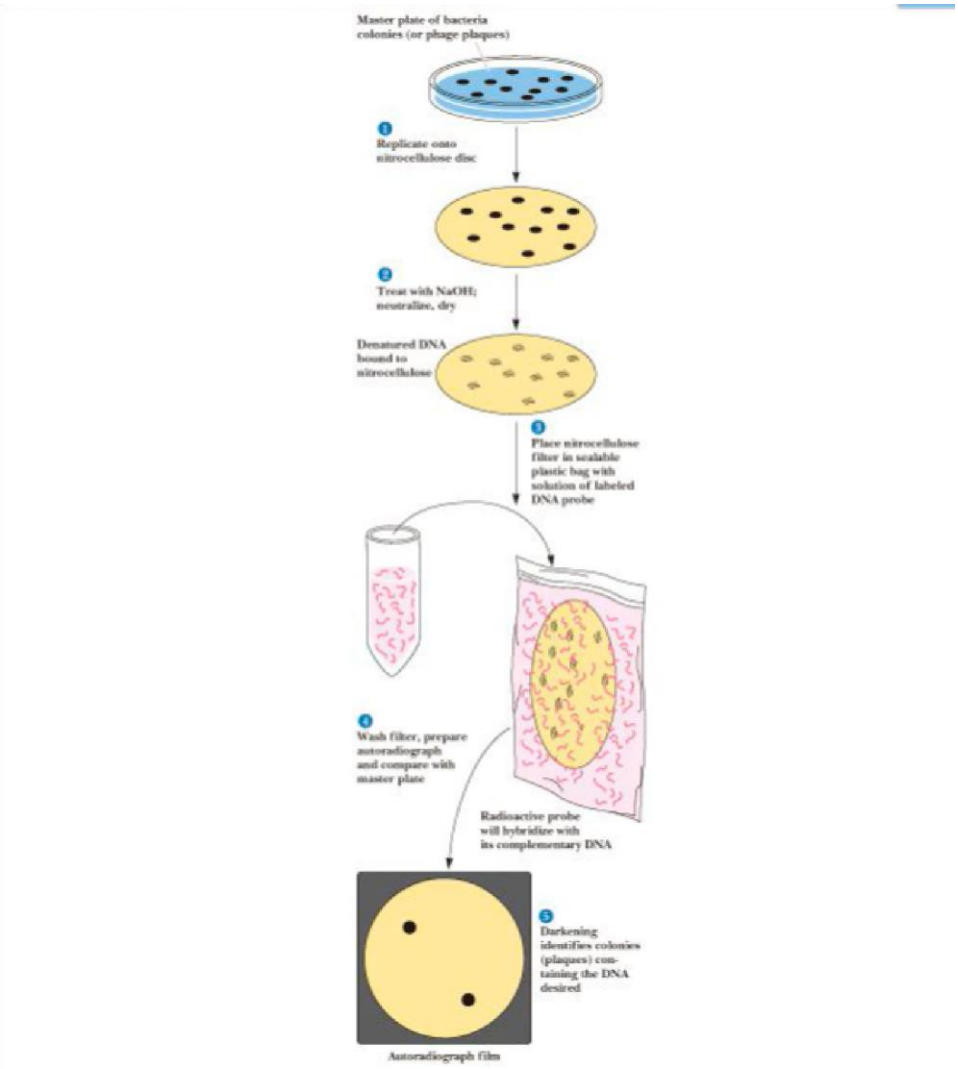

Figure 3.8: A colony hybridization experiment

DNA Libraries - Il

The probabilities are staggering!

- Consider the formula on page 406 for the probability of finding a particular fragment in N clones
- Suppose you seek a 99% probability of finding a given fragment in N clones of 10 kbp fragments
- If your library is from the human genome, you would need 1,400,000 clones to reach a 99% probability of finding the fragment of interest!

Colony Hybridization

A way to screen plasmid-based genome libraries for a DNA fragment of interest

- Host bacteria containing a plasmid-based library of DNA fragments are plated on a petri dish and allowed to grow overnight to form colonies
- Replica of dish made with a nitrocellulose disk
- The disk is treated with base or heated to convert dsDNA to ssDNA and incubated with probes
- Colonies that bind probe (with P-32) hold the fragment of interest

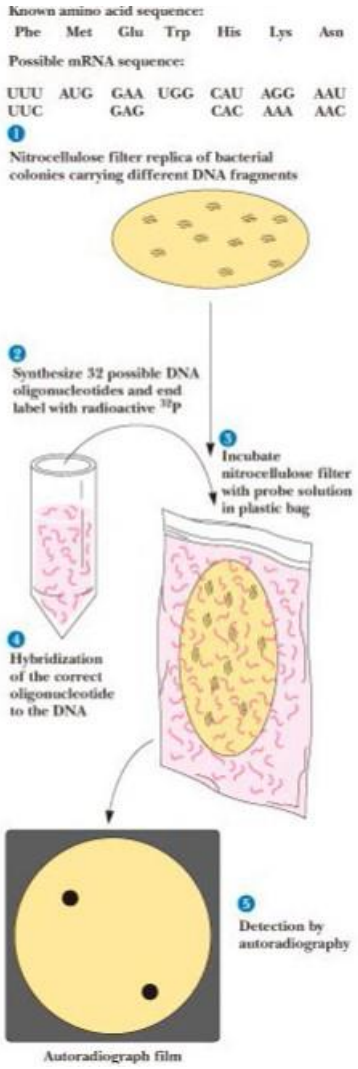

Figure 3.9: Cloning genes using oligonucleotide probes

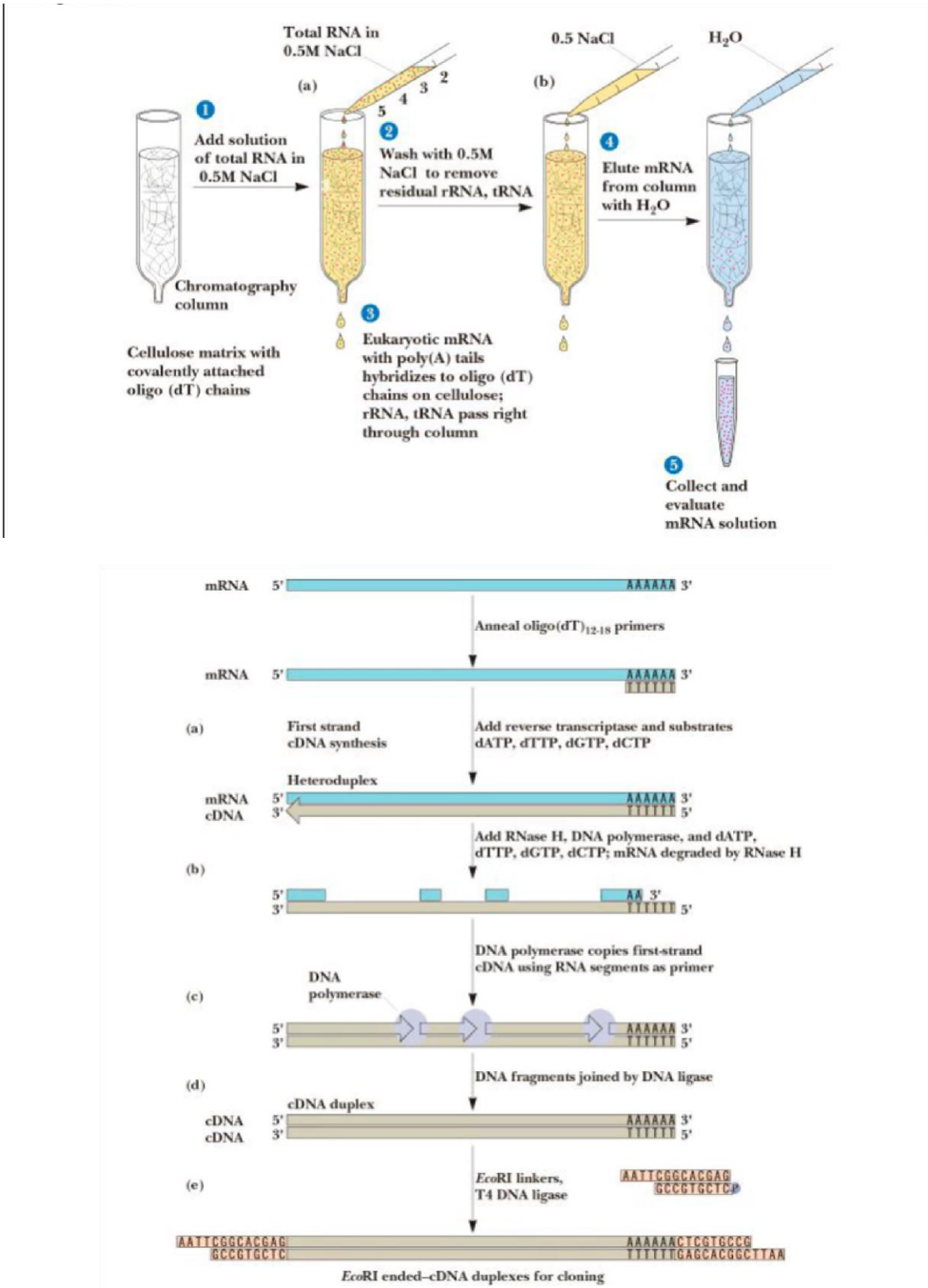

Figure 3.10: Isolation of eukaryotic mRNA via oligo(dT)-cellulose chromatography

Southern Blots

Another way to find desired fragments

- Subject the DNA library to agarose gel electrophoresis
- Soak gel in NaOH to convert dsDNA to ssDNA
- Neutralize and blot gel with nitrocellulose sheet
- Nitrocellulose immobilizes ssDNA Incubate sheet with labelled oligonucleotide probes
- Autoradiography should show location of desired fragment(s)

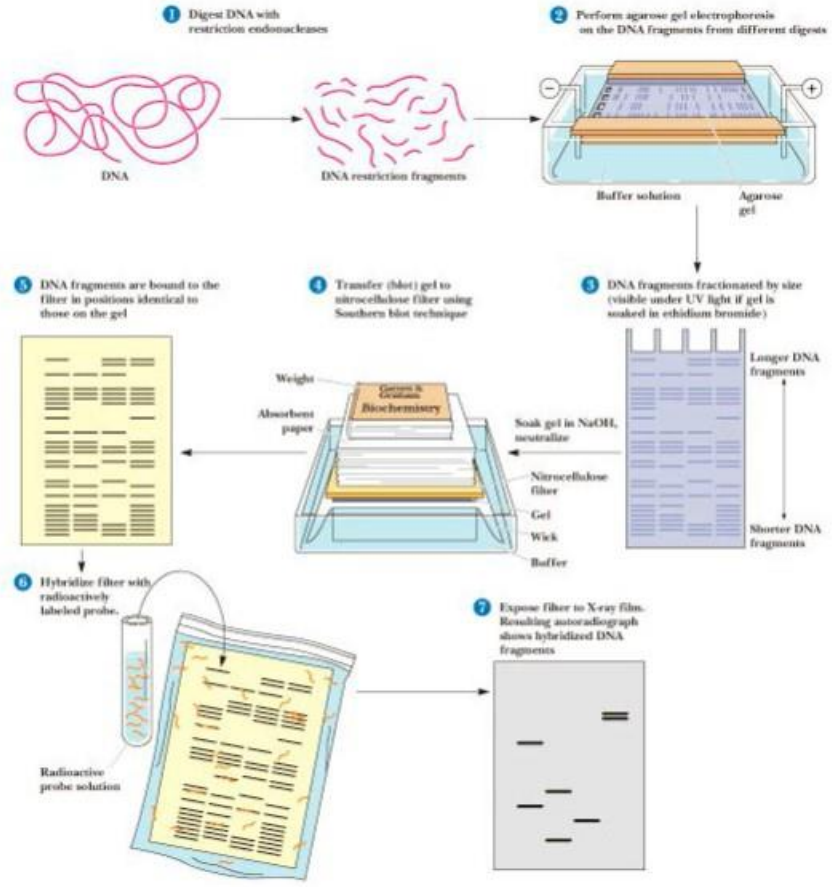

Figure 3.11: Southern Blotting

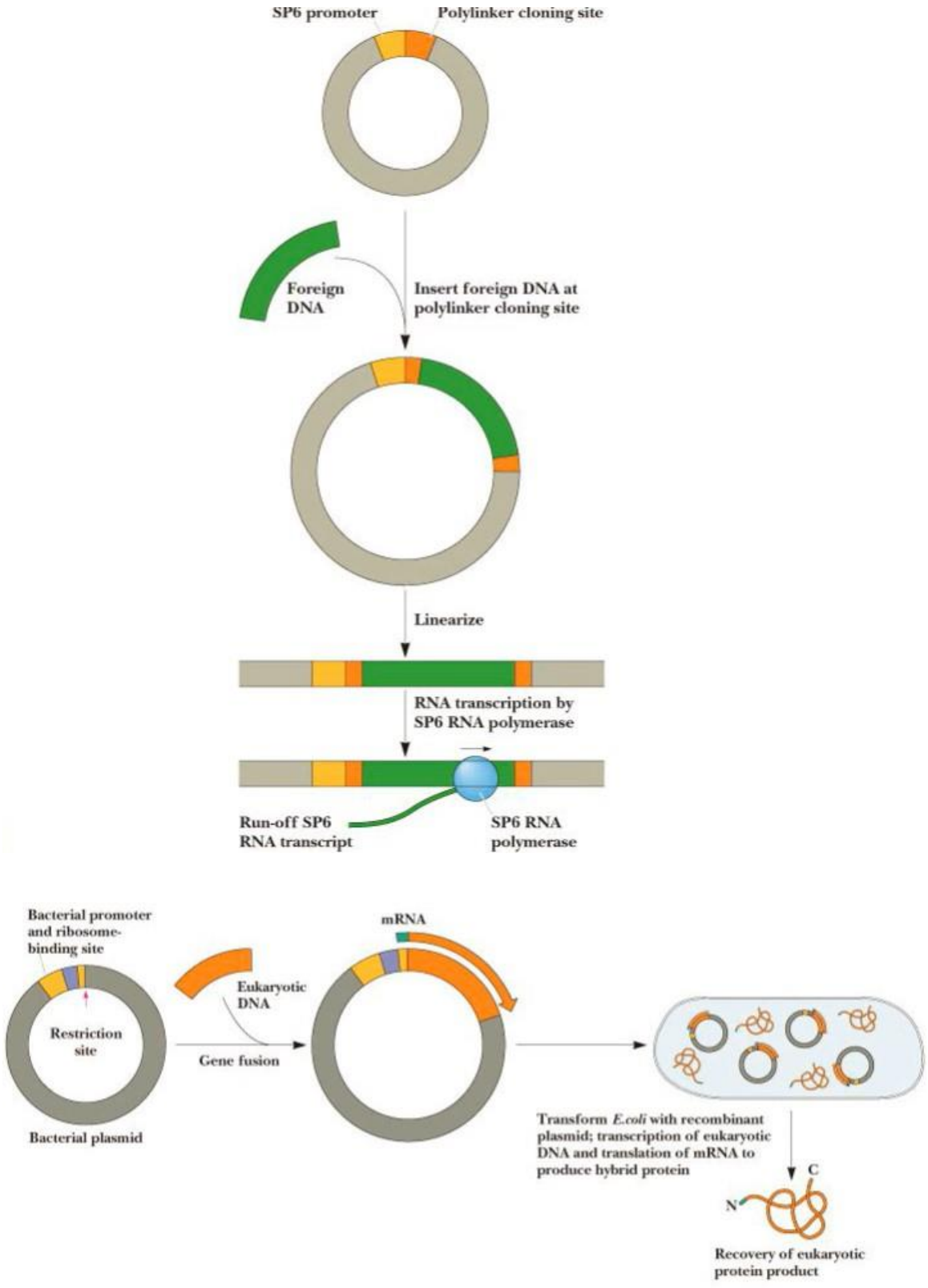

Figure 3.12: Expression Cloning Vector

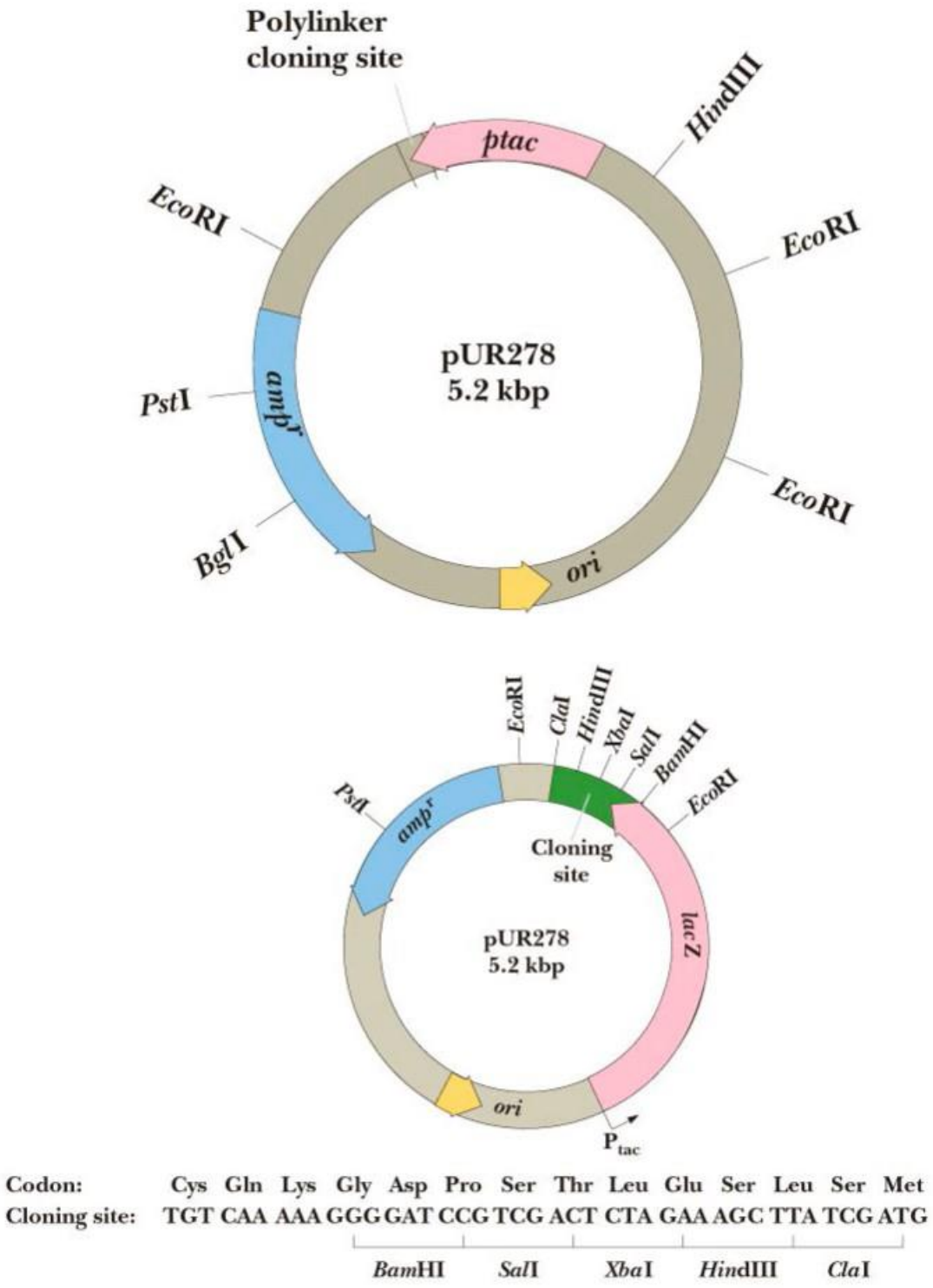

Figure 3.13: Expression Vector

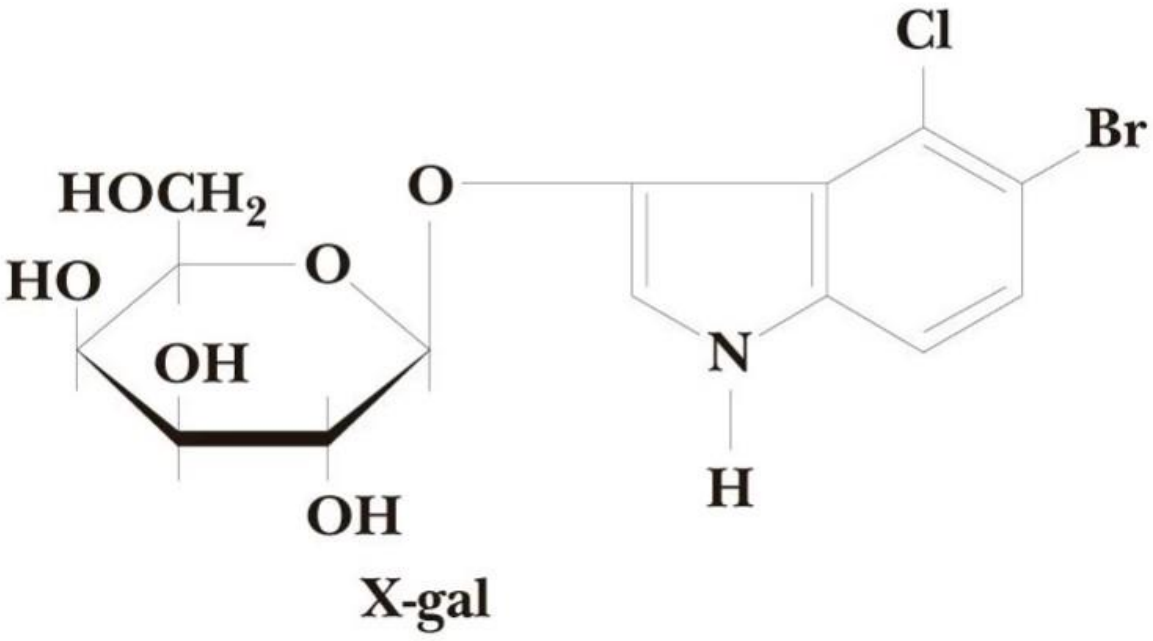

Figure 3.14: The structure of 5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside, or X-gal.

The Polymerase Chain Reaction

What if you don't have enough DNA for colony hybridization or Southern blots?

- The small sample of DNA serves as template for DNA polymerase
- Make complementary primers
- Add primers in more than 1000-fold excess
- Heat to make ssDNA, then cool
- Run DNA polymerase (usually Taq)
- Repeat heating, cooling, polymerase cycle

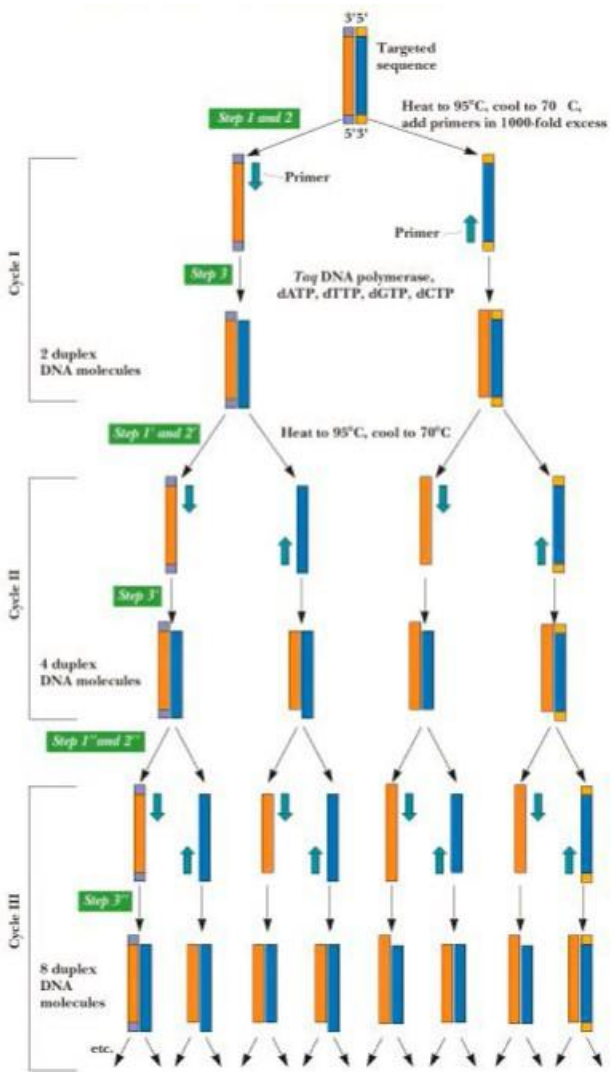

Figure 3.15: Polymerase chain reaction (PCR).

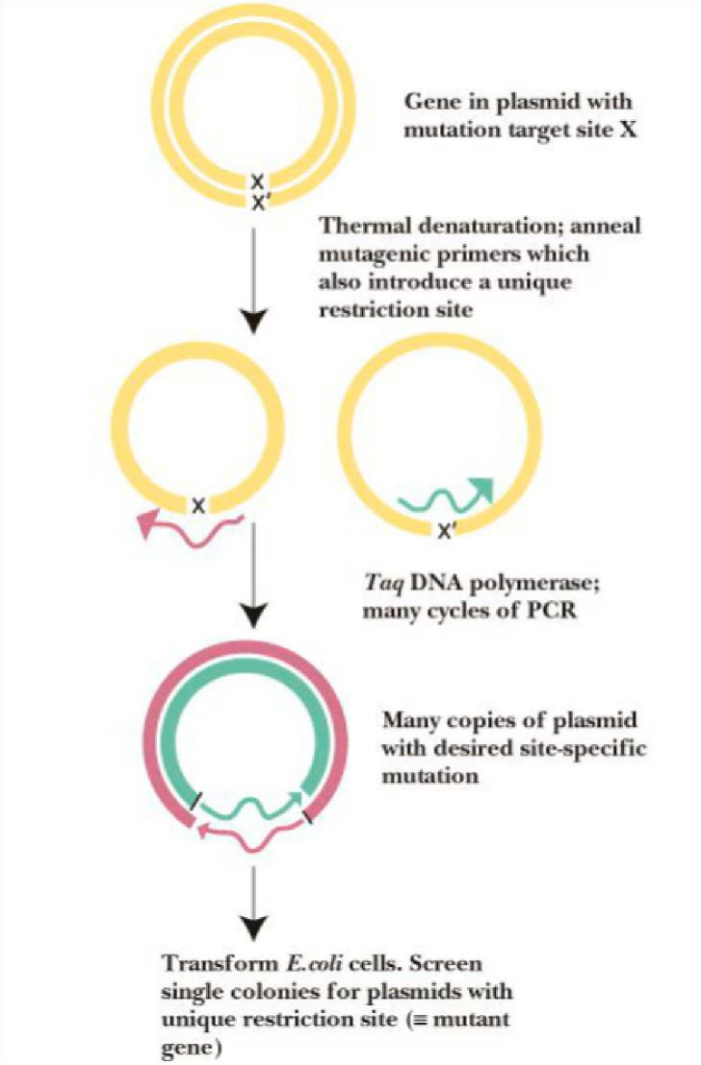

Figure 3.16: PCR-based site-directed mutagenesis.

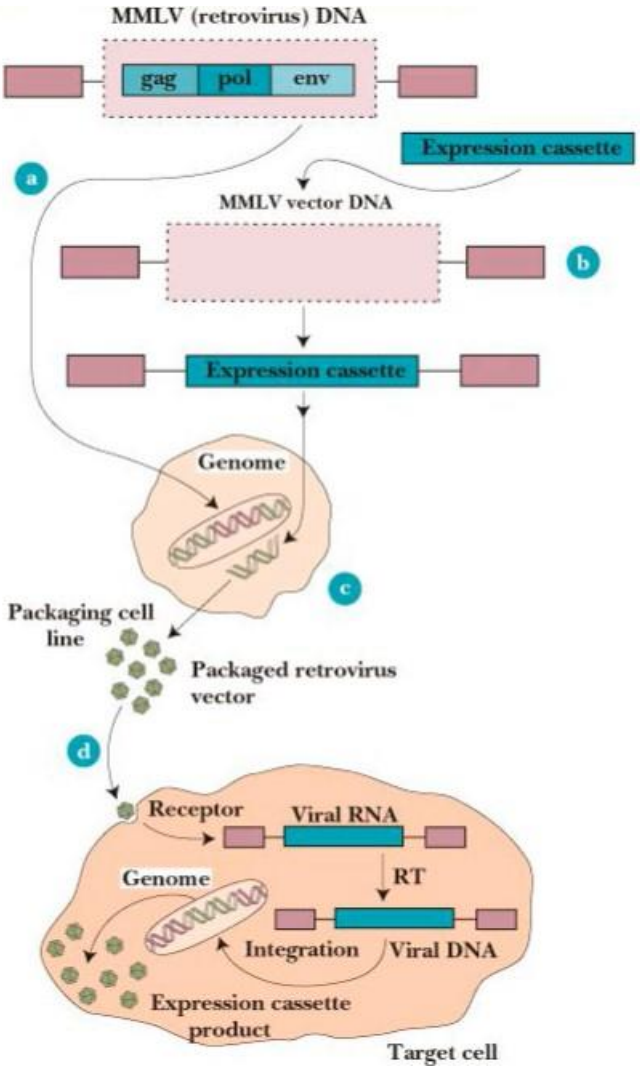

Figure 3.17: Retrovirus-mediated gene delivery

The **pronuclear microinjection** method introduces the transgene DNA at the earliest possible stage of development of the zygote (fertilized egg). When the sperm and egg cells join, the DNA is injected directly into the nucleus of either the sperm or the egg. Because the new DNA is injected directly, no vector is required; therefore there is no external genetic sequence to muddle the process

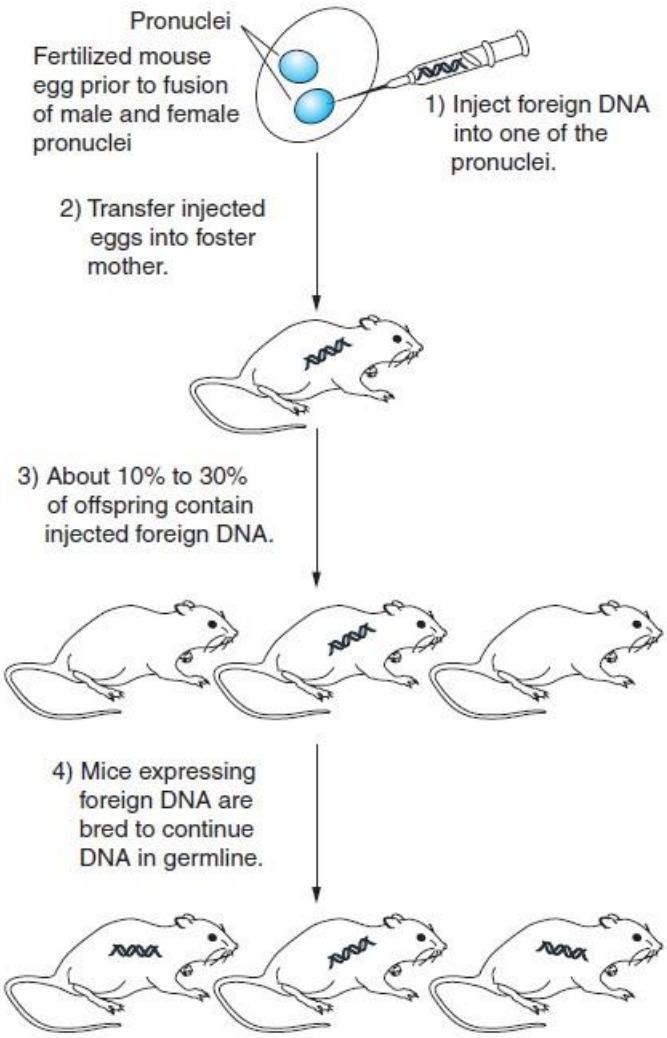

Figure 3.18: Pronuclear Microinjection Cloning

Gene Editing

Genome Editing: Three steps

- Target the locus.
- Delete or insert a gene.
- Repair the DNA strand.

What is Genome Editing based on?

The genetic material in every living cell is being constantly subjected to forces that drive genetic recombination and repair. Homologous recombination is driven by DNA modifying enzymes which include Restriction endonucleases, Recombinases, Polymerases and Ligases.

Genome editing harnesses these natural process to achieve the objective of modifying the genome in a precise and reproducible manner.

Process 1: Recombination

- Genetic recombination occurs during meiosis when genetic material is exchanged during the process of homologous recombination.
- The enzyme DNA recombinase initiates this process by catalyzing directionally sensitive DNA exchange reactions between short (30-40 nucleotides) target site sequences that are specific to each recombinase.
- RecA (bacterial)/ Rad51(H. sapiens) are involved in homologous repair and recombination in double-stranded DNA break repair. Rad51 protein performs the tricky task of pairing up a broken strand with its backup copy. The broken strand is first processed by other proteins to expose a single strand. Rad51 surrounds this single strand, and then captures the backup DNA copy, matching the sequence of the broken strand with a homologous sequence in the intact DNA double helix. Inside the Rad51 complex, the single strand is then exchanged for one of the strands in the duplex DNA, powered by ATP. Finally, a host of other proteins fill in all the missing sections of DNA, ultimately restoring two matching copies.

Process 2: DNA Restriction

Restriction endonucleases 'cut' or restrict the genome by identifying a specific set of sequences within the genome. In living bacteria, these enzymes serve as a means to defend the cell against invading bacteriophages. The restriction sites within the host genome are heavily methylated, this protects the host genome from digestion. A bacterium is immune to its restriction enzymes.

How can restriction enzymes be applied to genome editing in vivo?
The fragment of the restriction enzyme that is involved in the cleavage of the DNA molecule can be fused with Transcription Activator (TAL) proteins to develop TALENS.

Process 3: DNA Repair

- DNA polymerases play a critical role in the repair of the genome after the insertion or deletion of a gene irrespective of the genome editing strategy which is employed.
- DNA Ligases are involved in non-homologous recombination which occurs when genes are deleted.

TALEN
The Discovery of TAL

- Transcription Activator Like Effectors (TAL).
- Discovered in Xanthomonas.
- Injected into plant cells via the Type III secretion systems.

- Bind to promoter sequences of genes that facilitate bacterial infection.
- Specific binding to the DNA is based on a 34 amino acid repeat motif.

How Do TALs function?

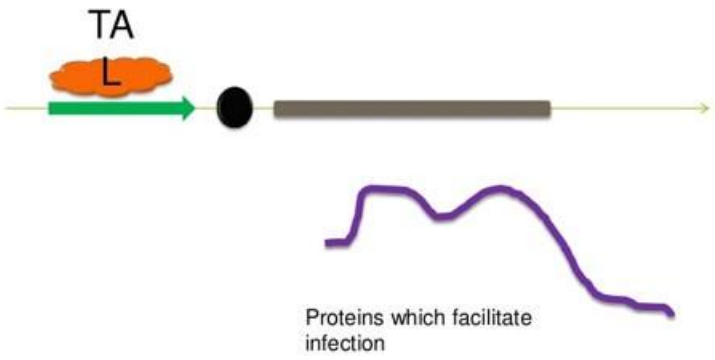

Figure 3.19: TALs Function

Typical Structure of TALS

- Central repeat domain.
- Number of repeats 1.5 to 33.5 ● Each repeat consists of 34 residues.
- Typical repeat sequence:
- LTPEQVVAIASNGGGKQALETVQRLLPVLCQAH G
- Short repeat at the Carboxyl terminal end.
- Hypervariable residues: positions 12 and 13. (Repeat Variable Domain).
- Structure: two alpha helices and a short RVD.

Typical Structure of TALS

Figure 3.20: TAL structure

This thirty-four amino acid domain is repeated (1.5) to (33.5) times within a typical TAL.

Amino Acid	Symbol
Asparagine	N
Histidine	H
Glycine	G
Isoleucine	I
Aspartic acid	D

How do we engineer TALS?

- Combinations of amino acids bind to specific nucleotides.
- This code has been deciphered.
- It is now possible to engineer TALs to target specific DNA sequences in vivo and in vitro.
- TALS can be fused to functional domains and applied to edit the genome.

TAL: Amino Acid – Nucleotide

Figure 3.21: TAL Amino acid

TALENs

Figure 3.22: TALENs

Constructing a TALEN

- TALENS (Transcription Activator Like Effector Nucleases) ● TAL = DNA Binding Domain.
- EN = Endonuclease of other DNA modifying domains.
- The endonuclease domain from the enzyme FokI is derived from the bacterium Flavobacterium okeanokoites.

Structure of a TALEN

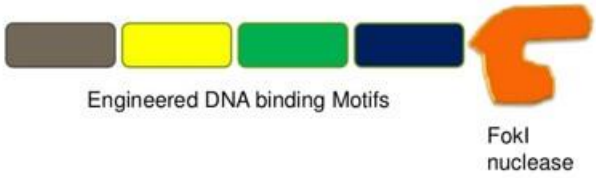

Figure 3.23: Structure of TALEN

The DNA binding motifs are engineered based on the coding amino acids in the TAL motif. The DNA sequence of the restriction enzyme FokI is fused to the DNA sequence for the TAL repeats resulting in the creation of a chimeric enzyme that can target specific regions of the DNA.

Engineering TALENs

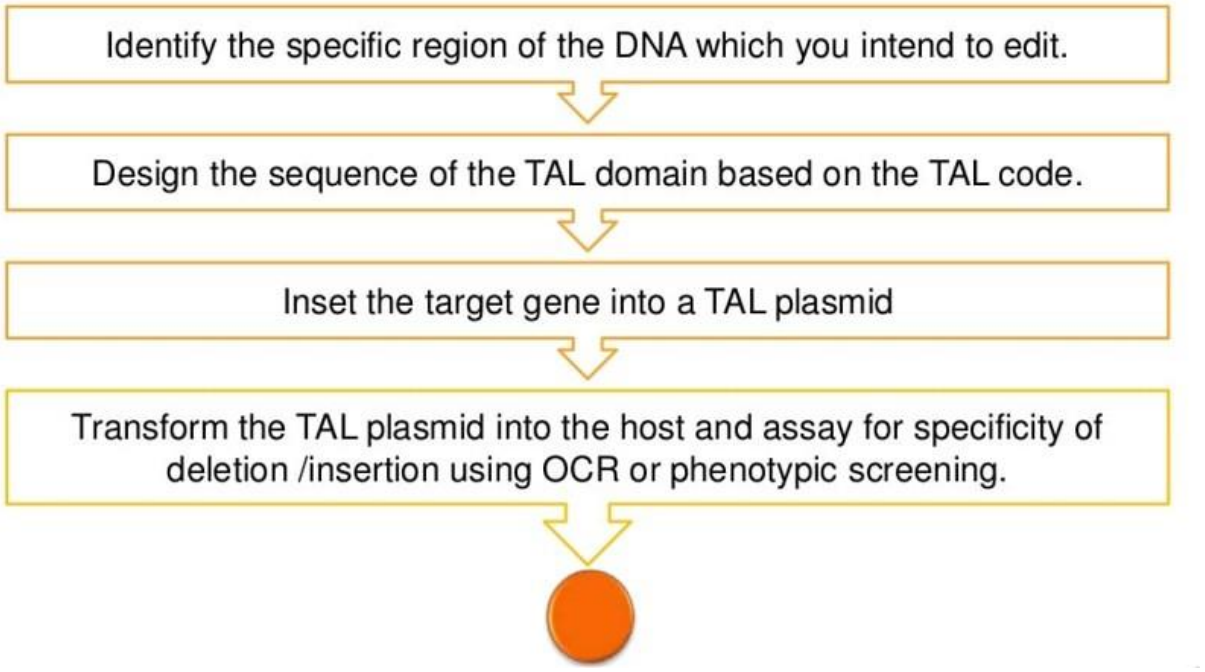

Figure 3.24: Engineering TALENs

How do TALENS work?

Figure 3.25: Working of TALENs

A pair of TALENs, one specific to each region flanking the targeted locus (black) are transfected into the cell and expressed in vivo. Upon expression the Fokl domain restricts the DNA on either side of the locus resulting in a double stranded break.

How do TALENS work: Deletions

Figure 3.26: Deletions

The DNA repair mechanism attempts to repair the gap by a process known as gap filling. It recruits DNA polymerases and DNA repair enzymes. However in the absence of either of the template strands, the gap is repaired without the gene. This results in a gene deletion.

How do TALENS work: Insertions

Figure 3.27: Insertions

If we introduce a DNA fragment with a homologous recombination site that matches the regions flanking the deletion, the DNA fragment integrates into the double stranded break. This results in a gene insertion.

CRISPR/Cas9

What is CRISPR?

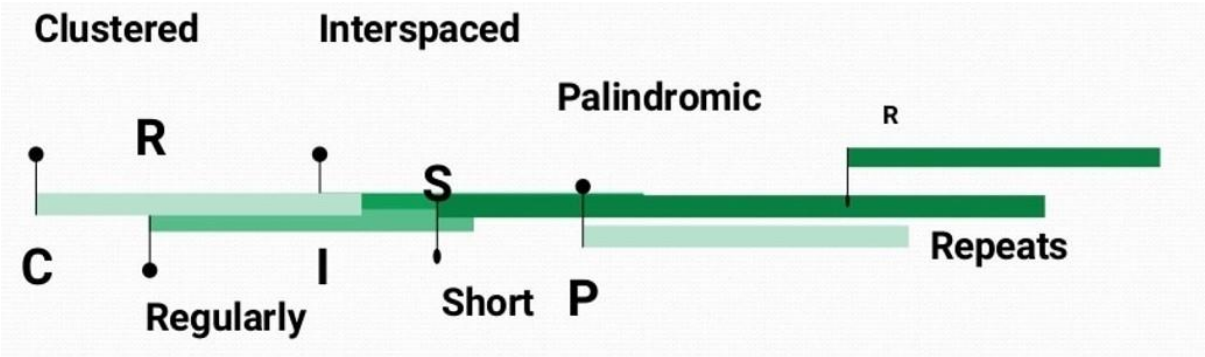

Figure 3.28: CRISPR

- CRISPR (krisper) is a family of DNA sequences in bacteria.
- The sequences contain snippets of DNA from viruses that have attacked the bacterium.
- These snippets are used by the bacterium to detect and destroy DNA from further attacks by similar viruses
- A palindromic repeat, the sequence of nucleotides is the same in both directions.
- Each repetition is followed by short segments of spacer DNA from previous exposures to foreign DNA (e.g., a virus or plasmid).
- Small clusters of cas (CRISPR-associated system) genes are located next to CRISPR sequences.
- The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity.

Evolution of CRISPR

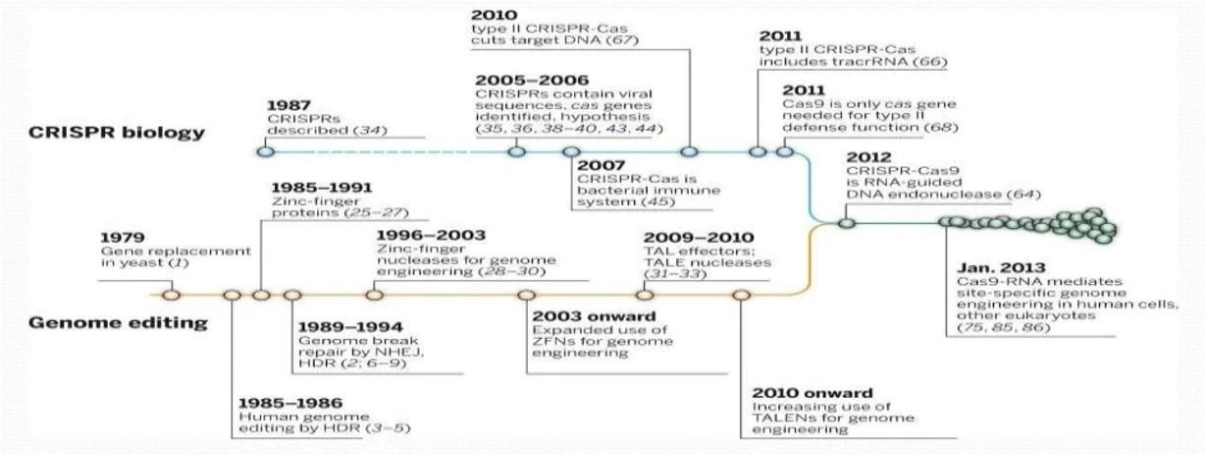

Figure 3.29: Evolution of CRISPR

CRISPR mode of action

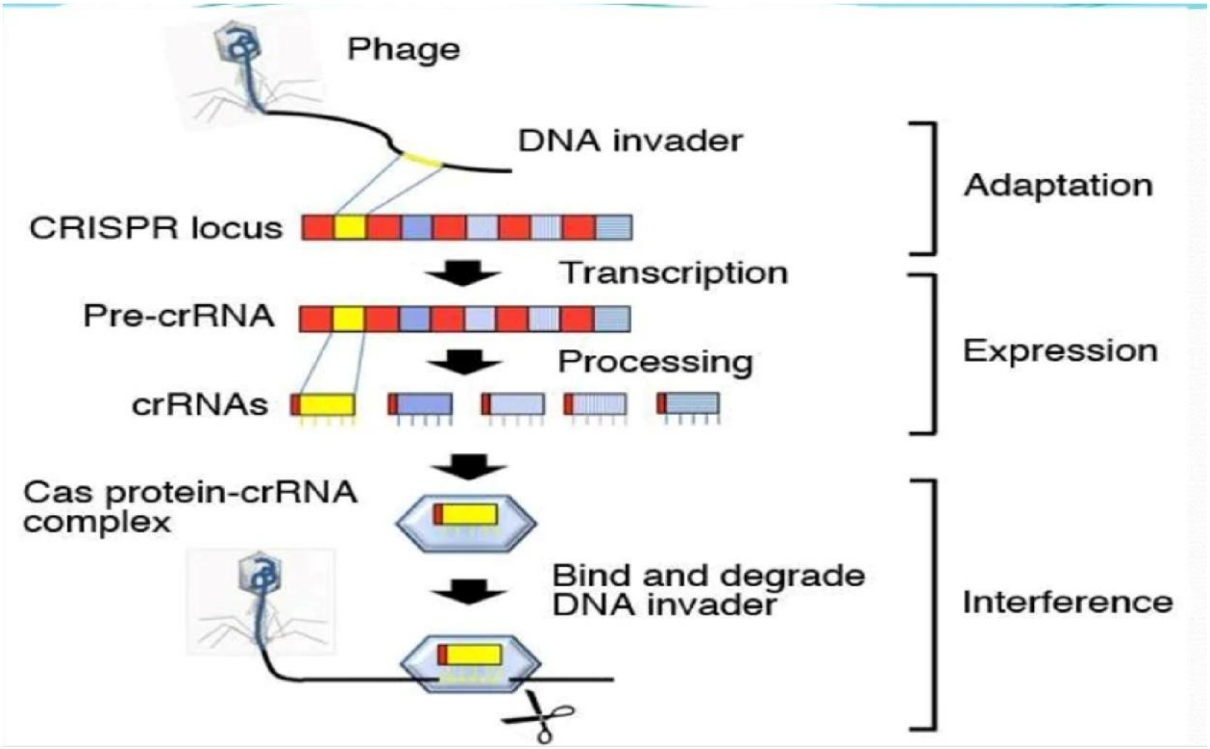

Figure 3.30: Mode of action

Three types of CRISPR systems

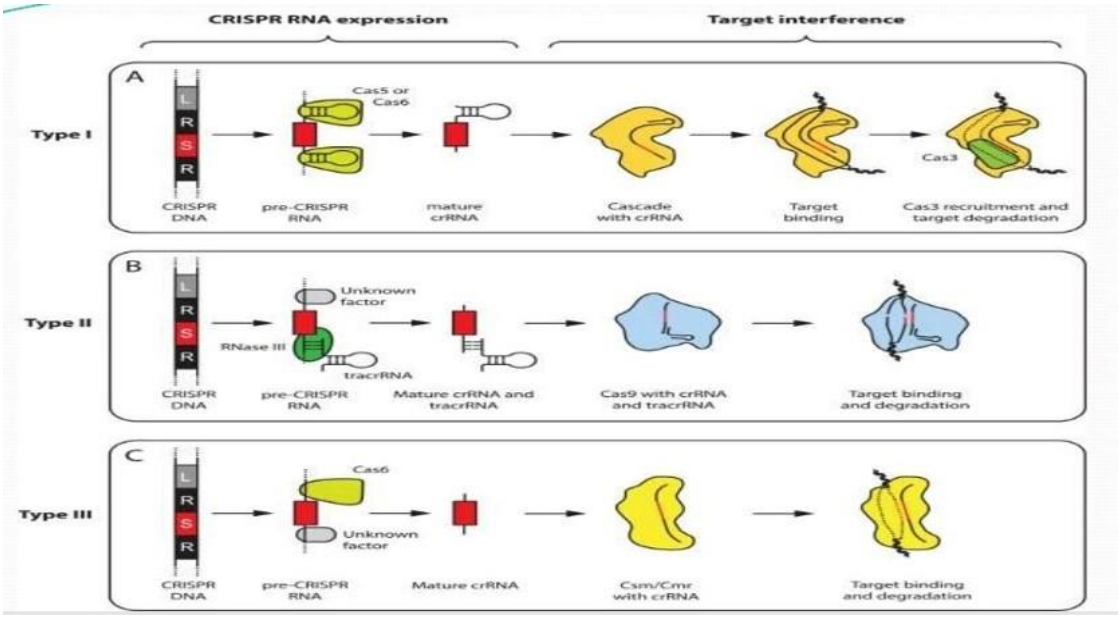

Figure 3.31: Three types of CRISPR

Cas-9 (CRISPR associated protein 9)

- is an RNA guided DNA endonucleases enzyme.
- associated with CRISPR
- which plays an role in adaptive immunity system, found in bacteria Streptococcus Pyogenes.
- involved in Type Il CRISPR mechanism

Biological structure of Cas-9

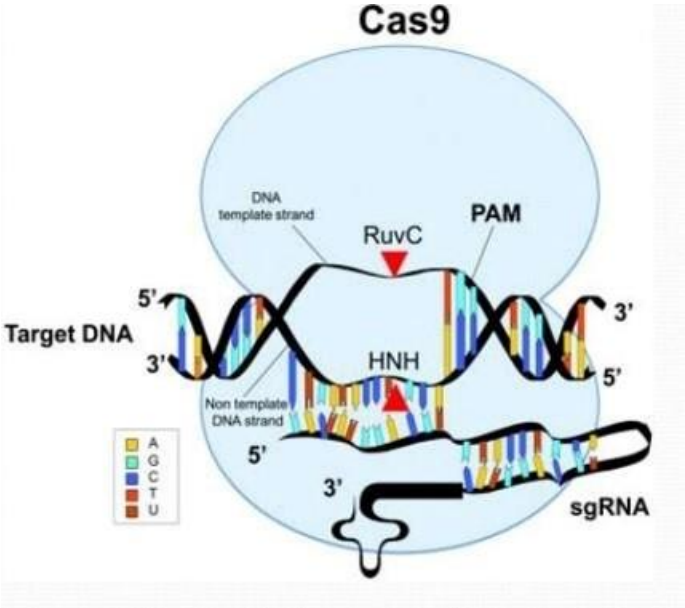

Figure 3.32: Biological structure of Cas-9

Cas9 protein has six domains

1. REC I-responsible for binding guide RNA
2. REC II-not yet well understood
3. Bridge Helix-(arginine-rich) is crucial for initiating cleavage activity upon binding of target DNA
4. PAM Interacting domain-confers PAM specificity responsible for initiating binding to target DNA
5. HNH and RuvCdomains -are nuclease domains that cut single-stranded DNA. are highly homologous to HNH and RuvC domains found in other proteins

3 types of Cas-9nucleases

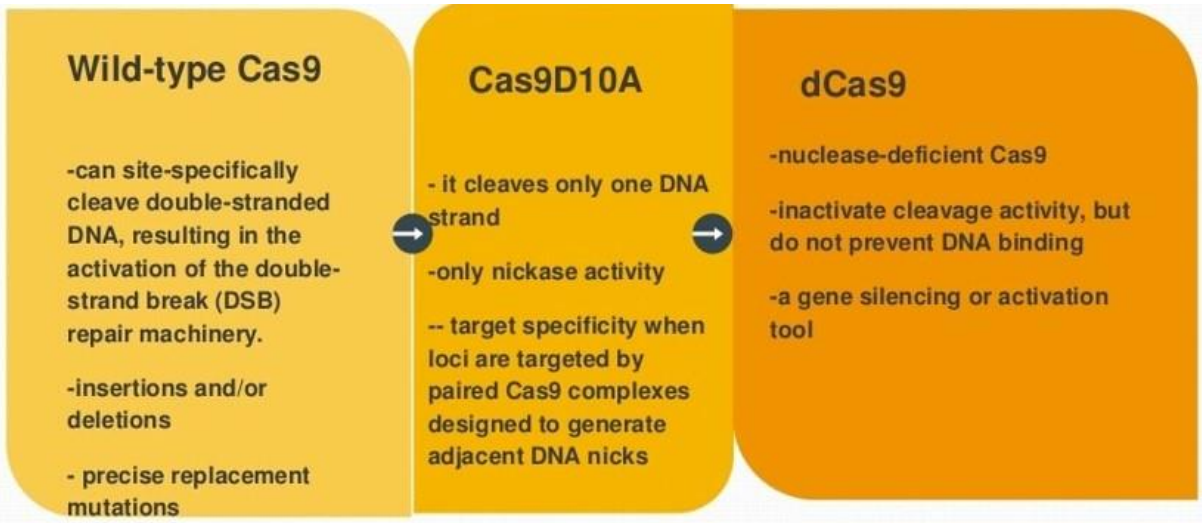

Figure 3.33: 3 Types of Cas-9 nucleases

Biologic Mechanism of action of Cas9

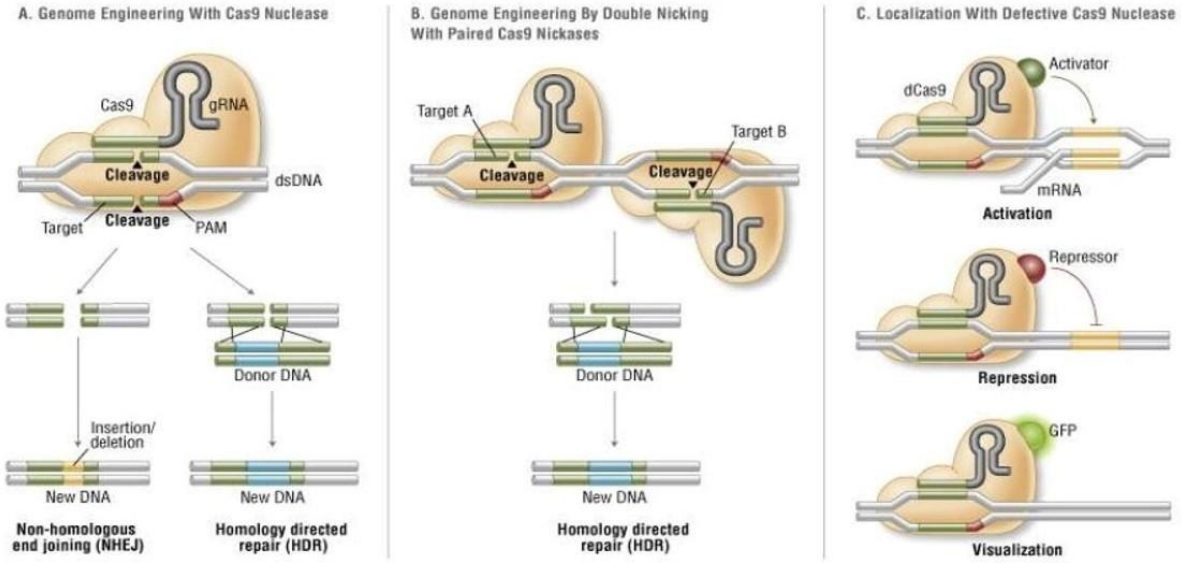

Figure 3.34: Biological Mechanism action of Cas9

Applications:

- Gene silencing
- DNA-free CRISPR-Cas9 gene editing
- Homology-directed repair (HDR)
- Transient gene silencing or transcriptional repression (CRISPRI)

- Transient activation of endogenous genes (CRISPRa or CRISPRon)
- Embryonic stem cell and transgenic animals ● Pooled genome-scale knockout screening

Implications:

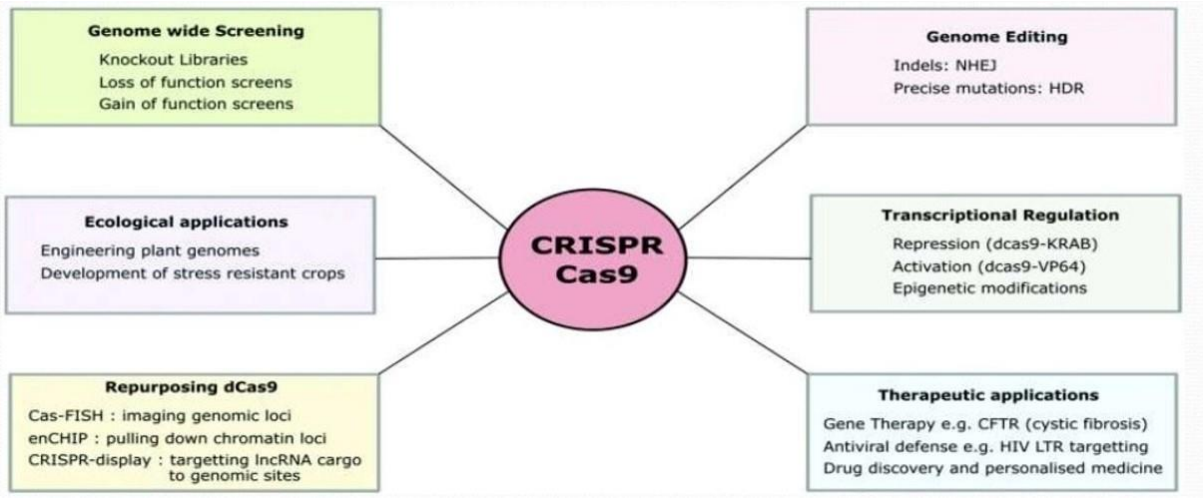

Figure 3.35: CRISPR Cas9

Clinical trials :

- The first clinical trial involving CRISPR started in 2016.
- It involved removing immune cells from people with lung cancer, using CRISPR to edit out the gene expressed PD-1, then. administrating the altered cells back to the same person.
- 20 other trials were under way or nearly ready, mostly in China, as of 2017.

Limitations:

- Targeting efficiency, or the percentage of desired mutation achieved, is one of the most important parameters by which to assess a genome-editing tool. ● T7 Endonuclease I mutation detection assay ● incidence of off-target mutations ???
- Recent improvements to the CRISPR system for reducing off-target mutations have been made through the use of truncated gRNA (truncated within the crRNA derived sequence) or by adding two extra guanine (G) nucleotides to the 5* end. Another method is use D10A Cas9 and two sgRNAs complementary ●

How far is targeting efficiency in humans???

- CRISPR Design Tool-webbased tools to facilitate the identification of potential CRISPR target sites and assess their potential for off-target cleavage.

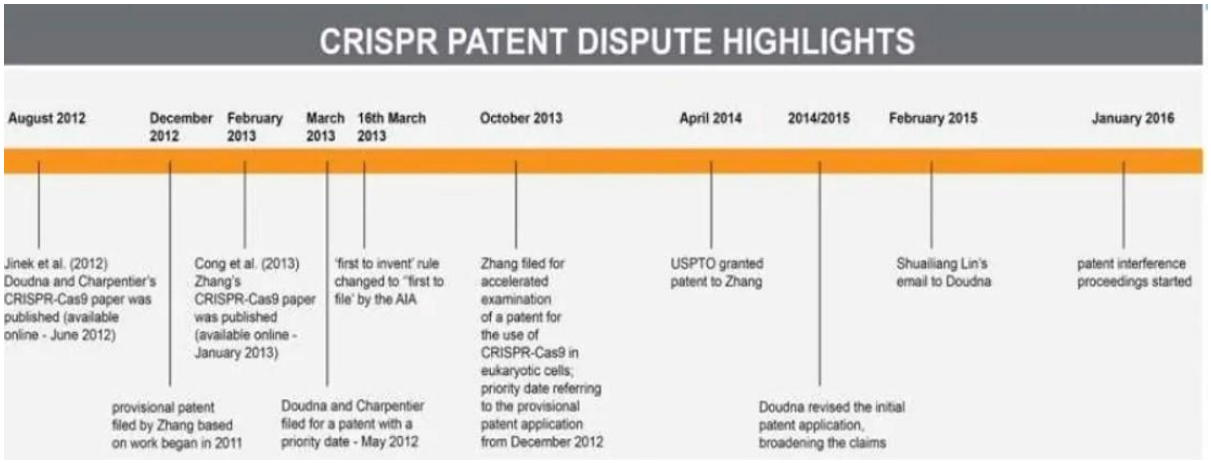

Figure 3.36: CRISPR patent dispute highlights

Importance of transgenic animals in Biotechnology

As disease model: Historically, mice have been used to model human disease because of their physiological, anatomical, and genomic similarities to humans. Transgenic animals are produced as disease models (animals genetically manipulated to exhibit disease symptoms so that effective treatment can be studied) such as Alzheimer's, cancer, AIDS. Transgenic animals enable scientists to understand the role of genes in specific diseases. The benefits of using transgenic animals include the possibility of the replacement of higher species by lower species- through the development of disease models in mice rather than in dogs or non-human primates, the extent of discomfort experienced by parent animals during the experimental procedures. Transgenic animals such as mice are valuable in investigations into gene function and for analysis of different hereditary diseases

Blood replacement Transgenic swine are used to produce human hemoglobin. The protein obtained from transgenesis could be purified by using procine blood which is similar to human hemoglobin.

Drug and Industrial production: Transgenic animals are used for the production of proteins such as alpha-1-antitrypsin, produced in the liver, used in the treatment of emphysema or cystic fibrosis. This process is less expensive than the production of protein through the culture of human cells. The human lungs are constantly get affected by foreign particles such as dust, spores, and bacteria. To prevent these, neutrophils release the elastase enzyme but this enzyme harmed the elastin in the lungs which maintains the elasticity of the lungs. So, the human body releases a protein α1 proteinase inhibitor which has been successfully expressed in sheep. Recombinant human proteins are produced in the mammary glands of transgenic animals.
Pharmaceutical proteins are now used for commercial purposes. Two scientists at Nexia Biotechnologies in Canada spliced spider genes into the cells of lactating goats. The goats are used to manufacture silk, milk and secrete tiny strands from their body by the bucketful. By extracting

polymer strands from the milk and weaving them into a thread which is a light and tough material that could be used to prepare military uniforms, medical micro sutures, and tennis racket strings. Americans are more supportive (60%) of the above use of transgenic animals. The mammary gland of transgenic goats is used to produce Monoclonal Antibodies. A recombinant bispecific antibody is produced by using transgenic cattle within their blood.
Another application includes the new generation of trans-chromosomal animals in which a human artificial chromosome containing the complete sequences of the human immunoglobulin heavy and light chain loci was introduced into bovine fibroblasts, which were then used in nuclear transfer. Transchromosomal bovine offspring were obtained that expressed human immunoglobulin in their blood. This could be a significant step forward in the generation of human therapeutic polyclonal antibodies

Adaptation of pig organs for transplantation to humans

Pigs have large litters, short gestation periods, and organs comparable to humans. **Pig heart valves** also have been used successfully for decades in humans. The blood thinner heparin is derived from pig intestines. Pigskin grafts are used on burns and Chinese surgeons have used pig corneas to restore sight.

Xenotransplantation: Now a day approximately about 250000 people are alive due to the successful transplantation of an appropriate allotransplantation. Sometimes there is the limitation of appropriate organs or rejection of live organ donation. So, to rectify this problem porcine xenografts from domesticated pigs are considered to be the best choice. Pigs that are genetically modified can be used as a source animal for tissues and organs in human beings for transplantation purposes by deleting the gene responsible for the human rapid immune rejection response. In Canada, a Nationwide survey on xenotransplantation showed that only 48% found it acceptable for 'the use of animals as a source of living cells, tissues or organs to prolong human life. To overcome the Hyperacute rejection & acute vascular rejection, synthesis of human regulators of complement activity are produced in transgenic pigs . Survival rates, after the transplantation of porcine hearts or kidneys expressing transgenic regulators of complement activity proteins to immunosuppressed nonhuman primates, reached near about 23 to 135 days. So, Hyperacute rejection can be overcome in a clinically acceptable manner. For long term graft tolerance induction of permanent chimerism via intraportal injection of embryonic stem (ES) cells or the co-transplantation of vascularized thymic tissue.

Disease control: Scientists developed the mice by altering the genes of the mousepox virus in Australia [50]. Some scientists also thought to develop genetically modified mosquitoes so they cannot produce malaria but other scientists worry about these mosquitoes that they could have unforeseen possibly risk if, they are released into the environment

Embryonic Stem Cell-Mediated Gene Transfer: In 1981, the term embryonic stem cells (ES cells) were used to denote a cell line isolated directly from mouse embryos while, the term

embryonal carcinoma cells (EC) were derived from teratocarcinomas. Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, including its gametes as shown in **figure 3.37**.

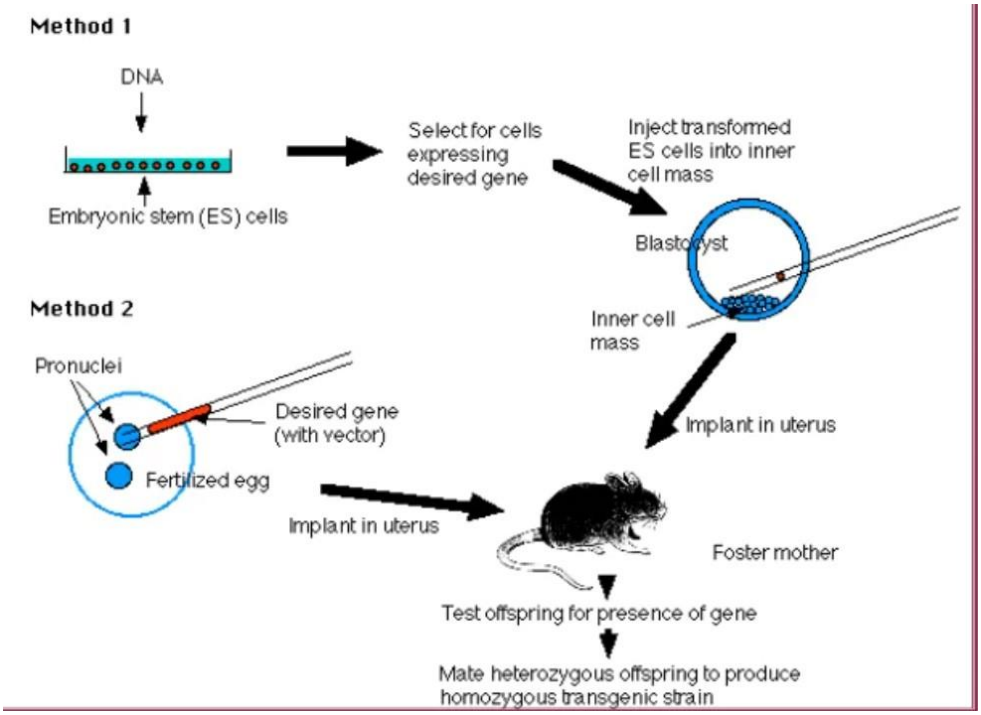

Figure 3.37: Embryonic Stem Cell-Mediated Gene Transfer

- Using recombinant DNA methods, build molecules of DNA containing the structural gene you desire (e.g, the insulin gene), vector DNA to enable the molecules to be inserted into host DNA molecules, promoter, and enhancer sequences to enable the gene to be expressed by the host cells.
- Transform ES cells in culture to expose cultured cells to the DNA so that some will incorporate it.
- Select for successfully transformed cells.
- Inject these cells into the inner cell mass (ICM) of mouse blastocysts.
- Embryo transfer.
- Prepare a pseudopregnant The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they implant successfully and develop into healthy pups (no more than one-third will).
- Test her offspring.

    - Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10- 20% will have it, and they will be heterozygous for the gene.
- Establish a transgenic strain o Mate two heterozygous mice and screen their offspring for the 1:4 that will be homozygous for the transgene. o Mating these will find the transgenic strain.

Retrovirus mediated gene transfer: Transgenic mice produced by retroviral transduction of male germline stem cells. Male germline stem cells can self-renew and genetic modification of these cells would help to study the biology of their complex self-renewal and differentiation processes and to generate a wide range of transgenic animal species. A retrovirus is a virus that carries its genetic material in the form of RNA rather than DNA. Retroviruses are used as vectors to transfer genetic material into the host cell, resulting in a generation of chimera (an organism consisting of tissues or parts of the diverse genetic constitution). Chimeras are inbred for as many as 20 generations until homozygous (carrying the desired transgene in every cell) transgenic offspring are born. The method was successfully used in 1974 when a simian virus was inserted into mice embryos, resulting in mice carrying this DNA.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 4 – MANIPULATION OF EMBRYOS - SBTA 1601

UNIT 4: MANIPULATION OF EMBRYOS

What is Micromanipulation Technology?

- Micromanipulation refers to the procedures which are performed using microscopic instruments to manipulate oocytes (eggs), sperm, and embryos.
- These microscopic techniques are designed to increase the chances of a successful in vitro fertilization (IVF) cycle.
- Preimplantation genetic diagnosis (PGD), ICSI, and assist hatching are offered to patients who can benefit from these techniques.
- During fertilization inside the human body, normally every few sperm arrive at the egg. With IVF, many thousands of sperm reach the vicinity of the egg, greatly improving the chances of successful fertilization.
- During conventional in vitro fertilization (IVF), eggs from the woman are collected and placed in a petri dish or test tube containing many sperm cells.
- The gametes are then left in an incubator overnight for fertilization to occur.

Figure 4: Sperm cells

- When this method fails, or when only a few sperm cells are available, then assisting fertilization using micromanipulation needs to be applied.

Brief History:

- In 1966, Lin described the technique of micromanipulating and injecting a mouse egg
- Subsequently, transgenic animals have been produced by introduction of foreign genes at the pronuclear stages of fertilized, one-cell zygotes
- Most of the successes have been with mouse and recently successful production of transgenic rabbit, pig, sheep and goat have been shown
- This technique is a powerful tool for studying gene regulation and physiological functions of gene products in a normal host environment
- Micromanipulation is the technique whereby sperm, eggs and embryos can be handled on an inverted microscope stage, performing minute procedures at the microscopic level via joysticks that hydraulically operate glass microtools.
- The injection of a single sperm into the cytoplasm of the oocyte, or intracytoplasmic sperm injection (ICSI), provided a satisfactory solution to the problems of the assisted fertilization techniques developed earlier.

- In this procedure, a single sperm is first immobilized by touching the sperm tail with an injection pipette (inner diameter 5–7 μm). The injection pipette picks up the immobilized sperm, pierces the ZP and oolemma, and delivers the sperm inside the oocyte cytoplasm.
- In 1976 using hamsters as a model, Uehara and Yanagimachi were probably the first to report the injection of sperm into oocyte cytoplasm (ooplasm).
- It was later attempted on rabbit and human oocytes, although the first successful human pregnancy was not reported until 1992 by the Free University of Brussels' group in Belgium.
- Micromanipulation technology has enabled the reproductive biologist to overcome inefficient steps in mammalian fertilization, the production of chimeric animals through blastocyst injection with embryonic stem (ES) cells and the introduction of specific genes into the genomes of domestic and laboratory animals.
- This technology has also been used for the production of cloned animals and ES cell lineages from cloned embryos, using nuclear transfer. Moreover, micromanipulation is also used for microsurgical embryo biopsy to study the basic developmental biology of embryos during preimplantation development.

Equipment used in Micromanipulation:

- A micromanipulator is a device which is used to physically interact with a sample under a microscope, where a level of precision of movement is necessary that cannot be achieved by the unaided human hand.
- It may typically consist of an **input joystick**, a mechanism for reducing the range of movement and an output section with the means of holding a microtool to hold, inject, cut or otherwise manipulate the object as required.
- The mechanism for reducing the movement usually requires the movement to be free of backlash. This is achieved by the use of **kinematic constraints** to allow each part of the mechanism to move only in one or more chosen degrees of freedom, which achieves a high precision and repeatability of movement, usually at the expense of some absolute accuracy. Movement reduction can be performed by **mechanical levers**, hydraulically using pistons of different diameters connected by tubing containing non-compressible fluid, electronically using **stepper motors or linear actuators**, or combinations of techniques in one instrument. Mechanisms with different ranges of movement or variable reduction ratio may be incorporated in one instrument to allow coarse and fine positioning.
- Micromanipulators are usually used in conjunction with **microscopes**. Depending on the application, one or more micromanipulators may be fitted to a microscope stage or rigidly mounted to a bench next to a microscope. A typical application of micromanipulation is **human intracytoplasmic sperm injection**. Here, a spermatozoon measuring some 3 to 5 micrometres across is injected into an oocyte of approximately 100 micrometres in diameter, under the direct manual control of an embryologist.
- A **disposable glass micropipette** is fitted to a tool holder mounted on the output of the manipulator.

- Micromanipulators are also used in applications such as microelectronics to position test probes onto small to medium scale integrated circuits and hybrid devices, and patch clamp experiments in biological research.

Enrichment Of X And Y Bearing Sperms From Semen Samples Of Animals:

The sex of an individual is determined at fertilization and depends on whether the X-bearing ovum is fertilized by a X or Y-bearing spermatozoa. There are several methods for diagnosing the sex of embryos. (a)Sex chromosome identification
(b)Demonstration of H-Y antigen
(c)Assay of sex-linked enzyme (d)Blotting
DNA probes

Embryo sexing :

Before the implantation of embryo its sex should be detected from the biopsy sample. The principle for sexing is very common.The presence of Y chromosome makes the offsprings male and that of X makes female. The second method is the use of polymerase chain reaction (PCR) machine in sex detection. PCR amplifies DNA sequence of Y chromosome and reaction products can be seen directly. Handy side et al. (1989) isolated single blastomere from early embryo from a womb, amplified DNA sequences of Y chromosome and carried out embryo sexing before implantation into uterus. It is true that the PCR was first commercially implemented for embryo sexing of livestock.

Embryo splitting:

Embryo splitting is another cloning technique. Using microsurgery (surgery conducted under a microscope), an embryo is split while it still consists of only a few cells. Genetically identical individuals develop from each portion in the same way that identical twins are formed in nature. This technique has been used to successfully clone embryos and animals.

Enrichment:

- This technique involves the separation of X- and Y- bearing spermatozoa in small quantities based on the DNA content of the spermatozoa so that they can then be used in in vitro fertilization and Artificial Insemination.
- Before the spermatozoa are passed through the cell sorter, the sample is exposed to a nontoxic DNA dye that specifically binds to DNA to allow measurement of the DNA content of each spermatozoa.
- The cell suspension is then passed through an extremely fine nozzle that vibrates at high frequency causing the fluid stream to disperse into micro-drops which, optimally holds only one spermatozoa after the conditions are calibrated.
- When the stream of micro-droplets is passing through a UV laser beam, the already stained DNA will start to glow or emit fluorescence.

- The strength (intensity) of the florescence is measured by a detector, and if the florescence is in the range designated for the Y-bearing spermatozoa, that micro-drop is given an electric charge.
- Furthermore, if the intensity is within the range for X-bearing spermatozoa, the micro-drop is given an opposite electric charge.
- The charged micro-drops fall between two charged plates, resulting in two groups of droplets to be separated into different pools.
- Droplets that fall outside of the fluorescence range for X or Y are collected into a third group where the sex cannot be determined.

Artificial Insemination And Germ Cell Manipulations:

- As the male produces millions of sperms daily, the semen can be used to produce several off springs.
- This is made possible by artificial insemination (AI) of females.
- Artificial insemination is done on standing animals through a technique, known as rectal palpation.

Definition: A process by which sperm are collected from the male, processed, stored and artificially introduced into the female reproductive tract for the purpose of conception.

1) Allows for widespread use of genetically superior sires that are too expensive to farmers to purchase.
2) Provides for faster and increased genetic improvement in cattle for improved herd performance and productivity.
3) Accelerated progeny testing of sires to determine their genetic potential.

Advantages of AI:

- Allows for the elimination of dangerous dairy bulls from the farm. Reduces the number of bulls in the herd.
- Allows to use larger and heavier bulls on smaller animals without the danger of injury to the females.
- Introduction of new genetic material via importation of semen from outside the country (no expensive handling, quarantine, and shipping costs of live animals). Frozen semen can be stored and used long after the donor or sire is dead / injured sire that is unable to breed naturally. Allows for more efficient use of estrous synchronization.

Disadvantages of AI:

- Requires well trained operators and special equipments.
- Requires more time than natural services.
- Necessitates the operator to have knowledge of anatomy and physiology of reproductive system of animals.
- Lower fertility (May occur in case of Improper cleaning of the instruments and insanitary conditions).
- Difficult preservation and transportation of semen (Under severe climatic conditions like those prevailing in most parts of tropics).

Collection of Semen:

The semen collection area should be as close as possible to the semen evaluation laboratory (not more than 30 m).

Common methods for collecting semen are: 1.

Massaging vesicular gland and ampullae.

2. Electro-ejaculation.

3. Artificial vagina (AV).

Massage vesicular gland and ampullae Method:

- This method is the simplest technique of semen collection.
- The operator (wearing long sleeve gloves) gently inserts his lubricated hand/ fingers of forearm into the male's rectum, empty the feces, gently massage the seminal vesicles/ampullae by backward and downward strokes toward the urethra and a cloudy fluid containing spermatozoa will be expelled.
- This method is seldom used because of poor response (some bulls), and dirty semen collections (contamination from the prepucial hairs).
- Commonly used to collect semen from cock, turkey and dog.

Electro-ejaculation method:

- Ejaculation of semen is brought about by inserting a probe or electrode in male's rectum stimulating nerves of the reproductive system by gradually increasing voltage in rhythmic fashion for a short period.
- Many types of electro-ejaculators in the market which are either operated only on battery or battery cum-electric transistorized circuits (battery connected to electricity).

Preparation/precaution:

The bull should be restrained in a crush. The ground surface of the crush should provide good footing.

Advantages:

1. Semen can be collected from males that are too young or old or unable to mount due to weakened or injured legs.
2. No female or dummy is required for collecting the semen.
3. Less chance of bacterial contamination.

Disadvantages:

a) Highly technical and needs considerable skill and practice.

b) Semen generally gets contaminated with urine.

c) Some males resist to this method and refuse collection.

d) Sciatic nerves are temporarily affected during the operation.

EVALUATION OF SEMEN:

Parameter for evaluation of semen

1. Visual evaluation for volume, color, consistency/density, smell/odor and for presence of foreign material should be made and recorded.

2. Microscopic evaluation for motility: mass activity and individual motility is done using a phase contrast microscope.
3. Determination of concentration; sing either Hemocytometer or a calibrated photometer.
4. Evaluation of live /dead counts: Buffered Nigrosin eosin solution is mixed with a drop of semen and smeared on a glass.
5. Morphological studies for head and tail abnormalities, and acrosomal abnormalities.

SEMEN DILUTION / EXTENSION:

- The main reason for extending (diluting) semen is to increase the number of females serviced from one ejaculation.
- A normal ejaculate from a dairy bull contains 5 - 10 billion sperm which can be used to inseminate 300 to 1000 cows if fully extended (15 to 20 million spermatozoa per straw for deep frozen semen).
- Dilution rate depends on quality of the ejaculate, number of sperm cells, percent alive and mobility.

AI Equipment ARTIFICIAL INSEMINATION PROCEDURE IN COWS:

- Forceps/tweezers:- for removing straws from the tank.
- Thermometer:- for measuring temperature of water used for thawing straws.
- Sharp stainless steel scissors:- for cutting straws.
- Paper towels:- for cleaning vulva, and wiping of scissors and straws.
- Insemination gun:- to expel the semen in female reproductive tract ¬ Has three parts: hollow catheter, plunger and O-ring. ¬ Three types: 0.5ml straw guns, 0.25ml straw guns, and universal straw guns(0.25 ml and 0.5ml straws)
- Disposable plastic sheaths – for securing straws to gun using O-ring • Two types of sheath: a) Split sheathes are designed for use with O-ring guns. b) Non-split sheaths for use with spiral or Kombicolor guns.
- Disposable plastic insemination gloves:- for protection of both inseminator and animal. Split sheaths Non- split sheath Plastic inserts.

Embryo cloning:

- A clone is a population of cells or organisms derived asexually from a single ancestor.
- They are genetically identical to each other to their common ancestor.
- Cloning means the production of exact genetic replica copies of an individual.
- They can not be considered as an offspring but simply the copy of a given individual.
- Much work has been done on cloning in plants and microorganisms.
- However, the techniques used in plants can not be applied for animals.
- Moreover, many animals from a single genetically superior embryo can be produced.
- Still there is no method of finding out which embryos are capable of cloning.
- It is useless to clone an embryo if it is not superior.

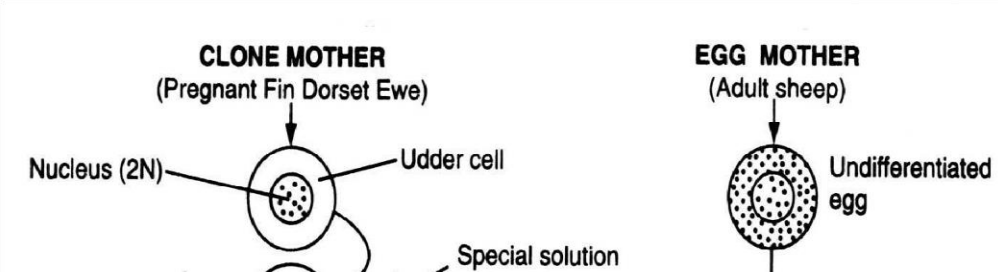
Quadriparental hybrid:

- During 1960s, Beatrice Mintz at Cancer Research Institute, Philadelphia (USA) demonstrated the interesting experimentation.
- She carried out fusion of embryos of two different species of mouse.
- This resulted in the formation of a single embryo which finally developed into a normal healthy animal having four parents.
- Embryo A was derived from the cross of male x female of one species, and embryo B derived after cross of male x female of the other species.
- The embryo A and B were united together and produced a single mass.
- In this experiment Mintz removed zona pellucida membrane of two early embryos and placed them in a suitable culture medium.
- The embryonic cells of the two embryos of blastula stage united randomly into a single mass of double sized embryo (blastocyst).
- A fresh membrane developed around the embryo.
- Then the embryo was transferred into the uterus of a foster mother.
- The foster mother was mated with a sterile male to bring her into proper stage for implantation. The first offspring with four parents was born in 1965.
- Similarly, exciting experiments have been done on another mammal.
- Following the same technique a hybrid of goat and sheep named geep was produced. At present two types of techniques for embryo cloning viz., nuclear transplantation (transfer) and embryonic stem cells, are being developed.

Nuclear Transplantation (Dolly):

- Nuclear transplantation (also nuclear transfer) involves removal of a single blastomere from a cleavage stage embryo with a fine micropipette of glass, and placing it under the outer membrane of an unfertilized mature enucleated oocyte (whose haploid nucleus has been removed by using micropipette or destroyed by UV light).
- For the first time in 1955, Robert Briggs and Tom King at Cancer Research Institute, Philadelphia (USA) carried out nuclear transplantation experiment on embryonic cells of frog. They transferred nucleus of undifferentiated blastula (a stage soon after fertilization of egg) into an enucleated egg cell.
- They noticed the normal development of the embryo.
- When they performed serial transplantation of differentiated nucleus from late gastrula (a stage after blastula) into a nucleus-free unfertilized egg, abnormal embryos were formed.
- This shows that cell nucleus is differentiated with embryo development.
- In 1960s, J.B. Gurdon at Oxford University, U.K. transferred differentiated intestinal nucleus of a frog into nucleus-free unfertilized egg of different amphibian species *(Xenopus laevis).*
- The embryo developed into tadpole and matured into frog (Gurdon, 1962). This new enucleated cell developed into normal embryo.
- Any damage to the donor nucleus during transplantation leads to abnormal development.

DOLLY:



- The first mammalian clone.
- 'Dolly', the worlds' first mammalian clone has been created from a fully differentiated non-reproductive cell of an adult sheep.
- It was born in February, 1996.
- The name Dolly has been given after an American country singer, Dolly Parton.
- In 1995, Ian Wilmut and his team of researchers at Roslin Institute, Edinburgh, Scotland, took udder (a fully differentiated tissue) from six year old sheep, Fin Dorset Ewe, and placed it in special solution that controlled cell cycle of cell division.
- The cell was deprived off certain nutrient. At the same time an unfertilized egg was obtained from another adult sheep.
- Its nucleus was carefully removed leaving the intact cytoplasm in egg.
- The nucleus of udder cell was taken out and transferred into nucleus-free egg.
- This was facilitated by applying mild electric sock.
- The newly transplanted nucleus soon became functional according to the new cytoplasm in which it had been artificially transferred.
- This viable combination underwent cleavage like normal zygote.
- This so-called embryo was then transplanted into the uterus of a third adult sheep (surrogate mother/foster mother) for its further development.

Wilmut's cloning experiment showing the birth of Dolly through nuclear transplantation technique.

- Finally, a normal healthy little lamb, Dolly was born in February, 1996 which was genetically similar to the *clone mother* from which nuclear DNA was taken out.
- It does not have any similarity with that sheep from which egg was taken out or surrogate mother because they did not contribute any genetic character.
- Thus, Dolly has only a single parent because she has born asexually, a characteristic feature found in lower forms of animal life, not in mammals.

Although behind this great success the rate of success is very slow, yet it has given some hope to embryo-biotechnologists to bring about refinement.

- Out of 277 nuclei transferred singly to enucleated egg, only 29 eggs grew into embryos.
- Out of these, only 13 embryo could be successfully transplanted into surrogate mothers.
- Of these only one ewe was successful in giving birth to an offspring, Dolly.

The significant considerations that can be derived from this experiment are that:

(i) The genes of differentiated cells have inherent totipotency

(ii) The interplay between the regulatory system of the genome in nucleus and the cytoplasmic factors of egg may make a cell totipotent

(iii) Possibly the cytoplasm of enucleated egg makes the transplanted nucleus totipotent like that of normal fertilized egg nucleus.

(iv) The maternally derived information in egg cytoplasm has an important role in cleavage which usually occurs after fertilization.

Production of chimeric mouse by embryonic stem cell transplantation.

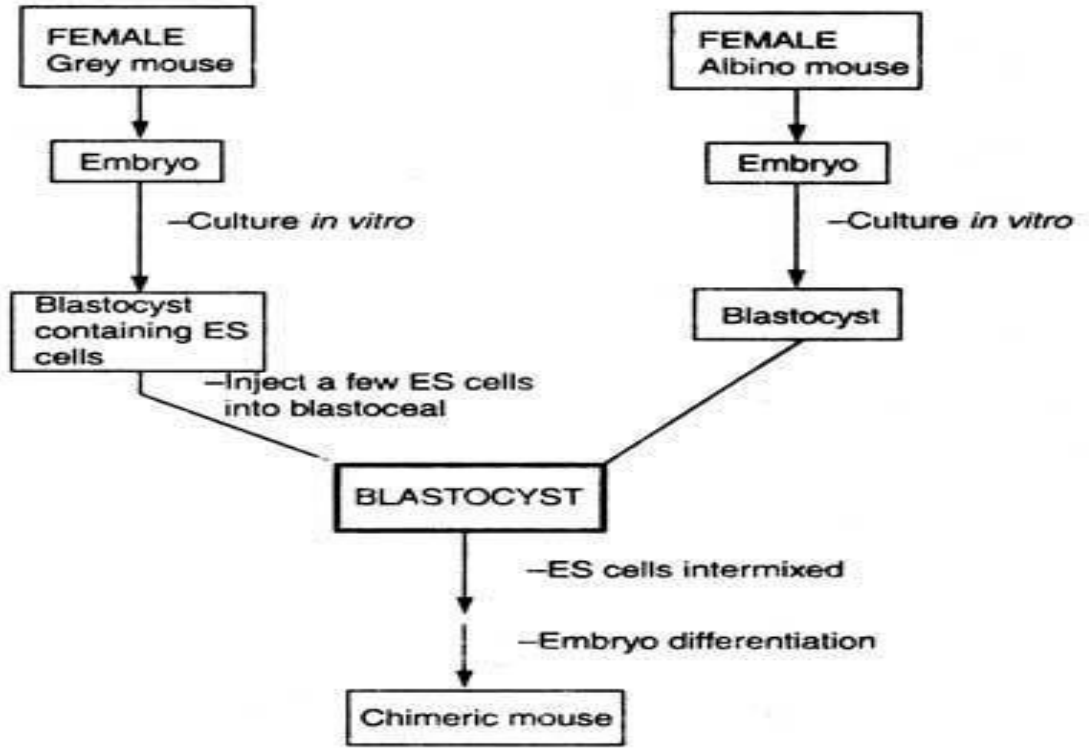


Figure 4.2: Stem Cell Transplantation

Hence, it is the egg cytoplasm but not the nucleus that regulates cleavage. Because the udder cell nucleus has limited potential for mitosis; it is the egg cytoplasm that interacted intracellularly with udder cell nucleus and stimulated to undergo repeated mitosis.

(v) carbon copy of the adult sheep could be produced without involving sperms from male partner.

(vi) the cloned animal produced via nuclear transplantation technique will be capable of restoring fertility as in 1998 Dolly gave birth to a little lamb named *Bonny.*

Embryonic stem cells:

- Embryonic stem cells (ESCs) are found in the inner cell mass of the human blastocyst, an early stage of the developing embryo lasting from the 4th to 7th day after fertilization.
- In normal embryonic development, they disappear after the 7th day, and begin to form the three embryonic tissue layers.
- ESCs extracted from the inner cell mass during the blastocyst stage, however, can be cultured in the laboratory and under the right conditions will proliferate indefinitely.
- ESCs growing in this undifferentiated state retain the potential to differentiate into cells of all three embryonic tissue layers.
- Research involving human ESCs is at the center of the ethical debate about stem cell use and potential in regenerative medicine.
- Embryos from which ESCs are extracted are destroyed in the process.

Embryonic Stem (ES) cells

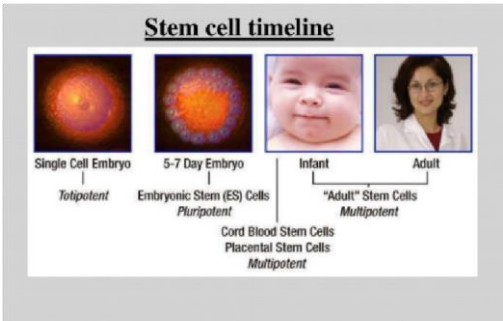


Figure 4.3: Stem Cell Timeline

Cloning of mice could not be done as in sheep via nuclear transplantation. This was due to acceleration of developmental programmes of embryo. However, it is evident that before first embryonic division the cell has started its process of differentiation.

Therefore, for cloning of mice an alternative approach has been made *i.e.* the use of ES cells. A blastocyst of mouse is place in culture condition. The inner cells that form future foetus continue to divide and remain in undifferentiated totipotent state as ES cells. There is a peptide growth factor known as leukaemia inhibitory factor (LIF) which establishes and maintains ES cell lines. The ES cell lines will be very useful in the area of production of transgenic animals *(see* preceding sections). However, the ES cells are used in two different ways: a small number of ES cells can be injected into blastocoel space of a blastocyst (Fig. 7.5). The ES cells get mixed with inner mass of cells of blastocyst to produce a chimera mouse which is a mixture of two cell genotype having the patches of different colored fur. Crossing of male and female chimera will allow selection of homozygous mice derived from ES cells.

Invitro Fertilization And Embryo Transfer:

Introduction:

IVF is the basic assisted reproduction technique, in which fertilization occurs in vitro (literally in glass). The sperm and the egg are combined in a laboratory dish, and after fertilization, the resulting embryo is then transferred to the female's uterus. The first successful IVF offspring were rabbits born in 1959. The next success came with laboratory mice in 1968. In 1978, the first IVF baby, Louise Brown was born. Since that time, this procedure has been used with increased success rates to produce offspring from patients with various infertility problems. The first live calf was born in 1981.

Procedure of IVF:

The basic requirements for IVF are healthy ova, sperm that can fertilize and a uterus that can maintain pregnancy.

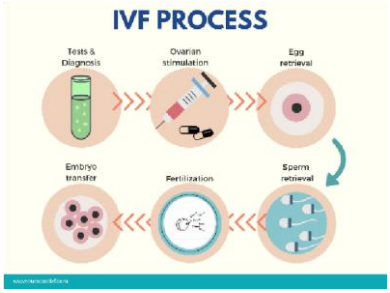


Figure 4.4: IVF Process

Five general steps are followed for IVF:

1. **Superovulation:**

Treatment cycles are typically started on the third day of menstruation and consist of a regimen of fertility medications to stimulate the development of multiple follicles of the ovaries. In most patients, injectable gonadotropins (usually FSH analogues) are used under close monitoring. Such monitoring frequently checks the estradiol level and by means of gynecologic ultrasonography, follicular growth. Typically approximately 10 days of injections will be necessary. Spontaneous ovulation during the cycle is typically prevented by the use of GnRH agonists, which block the natural surge f luteinising hormone (LH).

2. **Egg Retrieval:**

When follicular maturation is judged to be adequate, human chorionic gonadotropin is given. This agent, which acts as an analogue of luteinising hormone, would cause ovulation about 42 hours after injection, but a retrieval procedure takes place just prior to that, in order to recover the egg cells from the ovary. The eggs are retrieved from the patient using a transvaginal technique involving an ultrasound-guided needle piercing the vaginal wall to reach the ovaries. Through this needle, follicles can be aspirated, and the follicular fluid is handed to the IVF laboratory to identify

ova. It is common to remove between 10 and 30 eggs. The retrieval procedure takes about 20 minutes and is usually done under conscious sedation or general anesthesia.

3. **Fertilization:**

In the laboratory, the identified eggs are stripped of surrounding cells and prepared for fertilization. In the meantime, semen is prepared for fertilization by removing inactive cells and seminal fluid. If semen is being provided by a sperm donor, it will usually have been prepared for treatment before being frozen and quarantined, and it will be thawed ready for use. The sperm and the egg are incubated together,(at a ratio of about 75000 : 1) in the culture media for about 18 hours. In most cases, the egg will be fertilized by that time and the fertilized egg will show two pronuclei. In certain situations, such as low sperm count or motility, a single sperm may be injected directly into the egg using intracytoplasmic sperm injection. The fertilized egg is passed to a special growth medium and left for about 48 hours until the egg has reached the 6-8 cell stage.

4. **Selection:**

   Laboratories have developed grading methods to judge oocyte and embryo quality. Typically, embryos that have reached the 6-8 cell stage are transferred three days after retrieval. In many American and Australian programmes, however, embryos are placed into an extended culture system with a transfer done at the blastocyst stage at around 5 days after retrieval, especially if many good-quality embryos are still available on day 3. Blastocyst stage transfers have been in higher pregnancy rates. In Europe, transfers after 2 days are common.

5. **Embryo transfer:**

   Embryos are fraded by the embryologist based ont hr number of cells, evenness of growth and degree of fragmentation. The number to be transferred depends on the number available, the age of the woman and other health and diagnostic factors. In countries such as the UK , Australia and the New ZeaLand, a maximum of two embryos are transferred except in unusual circumstances. In the UK and according to HEPA regulations, a woman over 40 may have upto 3 embryos transferred, whereas in the USA, younger woman may have many embryos transferred based on individual fertility diagnosis. Most clinics and country regulatory bodies seek to minimise the risk of pregnancies carrying multiples. The embryos judged to be the best are transferred to the patient's uterus through a thin, plastic catheter, which goes through her vagina and cervix. Several embryos maybe passed into the uterus to improve chances of implantation and pregnancy.

Intracytoplasmic Sperm Injection:

Intracytoplasmic sperm injection iss an invitro fertilization procedure in which a single sperm iss injected directly into an egg. This procedure is most commonly used to overcome male infertility

problems, although it may also be used where eggs cannot be easily penetrated by sperm, and occasionally as a method of in vitro fertilization, especially that associated with sperm donation.
The procedure is done under a microscope using multiple micromanipulation devices (micromanipulator, microinjectors, micropipettes). A holding pipette stabilizes the mature oocyte with gentle suction applied by a microinjector. From the opposite side a thin, hollow glass micropipette is used to collect a single sperm, having immobilized it by cutting its tail with the point of the micropipette. The micropipette is pierced through the oolemma, the oolemma hardens to block the entry of any other sperm. Concern has been raised that in ICSI this sperm selection process is bypassed and the sperm is selected by the embryologist without any specific testing. However, in mid 2006 the FDA cleared a device that allows embryologists to select mature sperm for ICSI on sperm binding to hyluronan, the main component of the gel layer(cumulus oophorus) surrounding the oocyte. The device provides microscopic droplets of hyaluronan hydrogel attached to the culture dish. The embryologist places the prepared sperm on the breaks and significantly lower levels of aneuploidy than the sperm population from which they were selected. A brand name for one such sperm selection device is PICSI. There is some suggestion that birth defects are increased with the use of IVF in general and ICSI specifically, though results of different studies differ. In a summary position paper, The Practice Committee of the American Society of Reproductive Medicine has said it considers ICSI safe and effective therapy for male factor infertility, but may carry an increased risk for the transmission of selected genetic abnormalities to offspring, either through the procedure itself or through the procedure itself or through the increased inherent risk of such abnormalities in parents undergoing the procedure.

Preimplantation genetic Diagnosis:

Preimplantation genetic diagnosis refers to procedures that are performed on embryos prior to implantation, sometimes even on oocytes prior to fertilization. PGD is considered another way to prenatal diagnosis. Its main advantage is that it avaaoids selective pregnancy termination as the method makes it highly likely that the baby will be free of the diseaseunder consideration. PGD thus is an adjunct to assisted reproductive technology, and requires in vitro fertilization to obtain oocytes or embryos for evaluation. The term preimplantation genetic screening is used to denote procedures that do not look for a specific disease but use PGD techniques to identify embryos at risk. PGD is a poorly chosen phrase because, in medicine, to diagnose means to identify an illness or determine its cause. An oocyte or early-stage embryo has no symptoms of disease. They are not ill, rather they may have a genetic condition that could lead to a disease. To screen means to test for anatomical, physiological or genetic conditions in the aabsence of symptoms of disease. So both PGD and PGS should be referred to as types of embryo screening. Generally PGD is performed on an embryo which is in the 8 cell stage. A single cell is withdrawn using micromanipulators and is screened using either PCR or FISH.

Applications of IVF:

- Treatment of human or animal fertility
- Surrogate pregnancy
- Livestock improvement

- Preimplantation Genetic Diagnosis
- Harvesting of Embryonic stem cells
- Maintenance of genetic pool for long intervaals- Embryo cryopreservation
- Gene Transfer
- Increase in survivability of Endangered Species.

Advantages in Cell culture:

- Rapid Expansion of animal population ● Rescue of gene pool from dead/ frozen animals.
- Maintenance of lines with very low sperm count.
- Sperm- DNA co-incubation for genetic transformation
- Genetic manipulation is easy as mating of animals and harvesting of embryos is avoided.
- Large number of zygotes can be made at low cost.
- Embryos are free from viral diseases in infected parents.

Disadvantages of IVF:

- Not applicable in case of malformed eggs from female.
- Large offspring Syndrome.
- Embryo and Ovum damage due to manipulation ● Risk of Multiple gestation.

Ethical issues:

- Creation and discard of large number of embryos.
- Malicious Genetic / Sex selection in Humans.

Embryo Transfer Technology

The first successful transfer of fertilized rabbit eggs was reported in 1931 by Walter-Heape at Cambridge (UK). In farm animals embryo transfer was successfully done for the first time in 1934 in sheep and goats (Warwick et al., 1934). The first offspring after transfer of embryo in cattle and swine were reported in 1951 (Willet et al., 1951). Embryo transfer includes a sequence of various steps which are:

(a) selection of donors,

(b) induction of super ovulation

(c) embryos collection

(d) evaluation of embryos(e) selection of recipients and

(f) transfer of embryos.

Until now, embryo transfer techniques have been extensively used in cattle and more sporadically in sheep, goats and pigs. Selection of donor Regularly cycling cows or heifers can successfully respond to super-ovulatory treatment and be used for embryo transfer. The following selection criteria for donors will ensure a good probability of producing embryos of high quality.

1. Between the ages of 3 and 10 years

2. Free of genetic diseases and conformational abnormalities

3. Exhibit regular oestrus cycle

4. Shows superior production traits of economic importance

5. Free from infectious diseases.

6. Previous sound reproductive performances including no more than two inseminations per conception.

Induction of super ovulation Super ovulation means induction of multiple ovulation by application of exogenous hormones (PMSG-pregnant mare serum gonadotrophin; FSH follicle stimulating hormone; HMG-human menopausal gonadotrophin) in the early follicular or in the luteal phase of the oestrus cycle, in order to collect a large number of fertilized eggs. Most frequently PMSG or FSH is used. Only a single injection of PMSG (2000 to 3000 IU) is sufficient as the substance has a long half life time. In contrast, multiple injections of FSH (35-50 mg) are required to induce super-ovulatory responses (twice daily injections over four days). HMG (1000-1500 IU) also requires multiple injections; however, the drug is more expensive and only rarely used. Super-ovulatory response, embryo quality and fertility after treatment with different gonadotrophins in native Mertolengo cattle were studied (Lopes da Costa et al., 2001). In one experiment, super-ovulatory response (SR), embryo quality and plasma progesterone (P4) levels between donors treated with eCG treated donors. Fertilization rates were significantly higher in bred than in inseminated donors. Plasma P4 levels were only significantly different (higher) between responder and non-responder donors on the day of embryo recovery. Forty eight to sixty hrs after beginning the gonadotrophin treatment, prostaglandins (PGF2α) are administered to induce oestrus. Inseminations are performed at oestrus and the embryos are recovered 6-8 days after insemination. On an average, only one third of the super ovulated bovine donors show a good ovarian response. Embryo collection In bovines, embryos are recovered by non-surgical methods. Special catheters are introduced via the cervix into the uterine horn and embryos are flushed with 250 to 300 ml of flushing medium (phosphate buffered solution with 1-2% fetal calf serum). In the cow, the embryos usually enter the uterus on day four after oestrus. Non-surgical techniques avoid the potential damage of the reproductive tract by adhesions, are repeatable and do not require elaborate facilities. Embryos are collected by non-surgical methods 6-8 days after the onset of oestrus. Prior to day 5, the embryos may be in the oviduct and after day eight they may be hatched (escape from the zona pellucida), difficult to visualize and fragile. At day 7, bovine embryos are about 150-190 μ in diameter, are still within the zona pellucida and at late morulla or blastocyst stage of development. In case of other farm animals like sheep, pigs and goats, embryo recovery is performed by surgical methods. The abdomen of the donors is opened by midline cut and embryos are recovered by flushing oviduct and/or uterine horns. Embryos are collected 4-5 days after insemination or mating. Evaluation of embryos After recovering ova and embryos from the flushing medium, their further development capacity has to be evaluated. Morphological criteria

in embryo evaluation are shape, colour, number and compactness of cells, size of the perivitelline space, number and size of viscles and the zona pellucida. Embryos are classified into 4 groups:

(a) Excellent embryos – embryos in the appropriate developmental stage with a perfect morphology.

(b)Good embryos – embryos in the appropriate stage of development with slight morophological deviations.

(c)Degenerated and/or retared embryos.

(d)Unfertilized ova.

Selection of recipients:

The normal physiology and health conditions, the reproductive status, lack of any reproductive disorders, compatibility of the donor with respect to the size of the foetus and oestrus synchronization are of considerable importance. In bovine, it has been proved that heifers and young cows are best suited as recipients. The oestrus of donors and recipients should be synchronized within 24 hrs. Spell et al. (2001) studied the effects of corpus luteum characteristics, progesterone concentration, donor-recipient synchrony, embryo quality, type and development stage on pregnancy rates after embryo transfer. Of the 526 recipients presented for embryo transfer, 122 received a fresh embryo and 326 received a frozen embryo. Pregnancy rates were greater with fresh embryos (83%) than frozen-thawed embryos (69%). Pregnancy rates were not affected by embryo grade, embryo stage, donor recipient synchrony of the palpated integrity of the CL. There was a significant, positive simple correlation between CL diameter ort luteal tissue volume and plasma progesterone concentration. Transfer of embryos Special catheters are available to perform non-surgical transfer in cattle. It is important to transfer the embryos into the tip of the uterine horn without damaging the endometrium. In cattle, after superovulatory treatment 8-15 follicles are detected at oestrus and about 6-11 ovulations on the day of embryo recovery. The average number of embryos recovered is 5-10 representing 60-70% of the number of corpora lutea. Pregnancy rate after transfer of freshly collected embryos is 55-65%, and of frozen/thawed embryos recovered in dairy cattle. About 60-70 of these embryos are considered to be capable of further development. Statistically 25 to 3 calves per successful flushing can be produced. The oestrus cycle of the donor and recipient should be closely synchronized if transferred embryos are to survive. Pregnancy rates are generally reduced unless the embryo is placed in the lumen of the uterine horn on the same side of the corpus luteum**.**

Micromanipulation Technology And Breeding Of Farm Animals:

- Micromanipulation is the technique whereby sperm, eggs and embryos can be handled on an inverted microscope stage, performing minute procedures at the microscopic level via joysticks that hydraulically operate glass microtools.
- Embryo sexing Before the implantation of embryo its sex should be detected from the biopsy sample. The principle for sexing is very common.
- The presence of Y chromosome makes the offsprings male and that of X makes female.

- The second method is the use of polymerase chain reaction (PCR) machine in sex detection. PCR amplifies DNA sequence of Y chromosome and reaction products can be seen directly. Isolated single blastomere from early embryo from a womb, amplified DNA sequences of Y chromosome and carried out embryo sexing before implantation into uterus.
- It is true that the PCR was first commercially implemented for embryo sexing of livestock. Embryo splitting Embryo splitting is another cloning technique.
- Using microsurgery (surgery conducted under a microscope), an embryo is split while it still consists of only a few cells.
- Genetically identical individuals develop from each portion in the same way that identical twins are formed in nature.
- This technique has been used to successfully clone embryos and animals.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 5 – THERAPEUTICAL APPLICATION AND SAFETY GUIDELINES – SBTA 1601

Unit-5

Recombinant Cytokines and Their Use in the Treatment of Diseases

Recombinant cytokines are pharmaceutical analogs of endogenously produced cytokines and colony-stimulating factors and are used therapeutically to increase the activity of the immune system or the production of particular populations of blood cells.

- Hormone-like, small low molecular weight polypeptides.
- Maintain communication among cells to coordinate the immune response.
- Act synergistically or antagonistically thereby enhancing or suppressing their production ● Autocrine, paracrine, or endocrine in action.
- Causes tissue repair and provide resistance to infection

Recombinant cytokines are generally designed to enhance immunity either by achieving generalized immunostimulation or stimulating specific immune cells. These proteins have not only improved treatment options for several diseases but have also been associated with novel toxicities.

Some cytokines have been developed into protein therapeutics using recombinant DNA technology. Recombinant cytokines being used as drugs as of 2014 include:

- Bone morphogenetic protein (BMP), used to treat bone-related conditions
- Erythropoietin (EPO), used to treat anemia
- Granulocyte colony-stimulating factor (G-CSF), used to treat neutropenia in cancer patients
- Granulocyte-macrophage colony-stimulating factor (GM-CSF), used to treat neutropenia and fungal infections in cancer patients
- Interferon alfa used to treat hepatitis C and multiple sclerosis ● Interferon-beta used to treat multiple sclerosis ● Interleukin 2 (IL-2), used to treat cancer.
- Interleukin 11 (IL-11), used to treat thrombocytopenia in cancer patients.
- Interferon-gamma is used to treat chronic granulomatous disease[42] and osteopetrosis[43]

Recombinant cytokines are generally designed to enhance immunity either by achieving generalized immunostimulation or stimulating specific immune cells. These proteins have not only improved treatment options for several diseases but have also been associated with novel toxicities. Toxicities arising from the use of pharmacologic immunostimulation can be mechanistically interpreted as imbalances in cytokine signaling, either due to cytokinemia or to secondary events resulting in exaggerated production of endogenous cytokines. Therapeutic immunostimulation aims to enhance responses against infection or malignant cells by activating innate immunity, enhancing adaptive immune responses, or overcoming immunosuppressive signals mediated by the pathogen or tumor microenvironment. Taken as a whole, evaluation of immunostimulatory proteins in nonclinical models are generally predictive of responses in humans, with a few exceptions such as cytokine storm, first-dose response, tumor lysis syndrome (given their absence of tumors), and autoimmunity.

Interleukin-12 (IL-12) Case Study: IL-12, a heterodimeric produced predominantly by macrophages and other antigen-presenting cells, is a key regulator of cell-mediated immunity. Recombinant human IL-12 (rhIL-12) was subsequently shown to increase IFNγ production from NK and T cells, enhance NK/lymphokine-activated killer (LAK) cell cytotoxicity, and promote T-helper cell type 1 (Th1) immune responses both *in vitro* and *in vivo*. rhIL-12 was used in clinical trials for its therapeutic potential as an anticancer and anti-infectious disease agent. These studies demonstrated that prior single-dose administration of lower doses of IL-12 reduced the toxicity of subsequent, higher doses, and this priming could mitigate lethal toxicities observed in patients treated with a single dose [83,84]. Such a schedule-dependent effect on pharmacology and toxicity in humans was later confirmed in mice and non-human primates and is attributed to attenuated pharmacology with repeated exposure [85]. Because of this unique schedule-dependent phenomenon, careful attention to dose and schedule has been required to assure safe and effective clinical development of this cytokine.

Interferon-alpha (IFN-a) Case Study: Various forms of recombinant human IFNα have been marketed and used to treat chronic hepatitis C virus infection and cancer [82]. Interferons play a major role in the first steps of the response to acute viral infections, being mediators in the nonspecific cellular antiviral response that precedes the specific, immune-mediated, response. Specifically, the immunostimulatory activity of IFNα includes upregulation of antiviral gene expression [87–89], suppression of cell proliferation [90,91], enhanced cytotoxic activity of macrophages, NK cells, and neutrophils [92,93], and inhibition of viral replication [94,95]. Toxicities associated with chronic dosing in patients are diverse and include flu-like symptoms, gastrointestinal effects, fatigue, CNS disorders, including neuropsychiatric abnormalities, and sleep disorders. In addition, laboratory abnormalities, including anemia, thrombocytopenia, and neutropenia, are associated with chronic treatment and constitute one of the major reasons for treatment discontinuation or dose adjustment. Despite the emergence of neutralizing anti-interferon antibodies in nonhuman primates (NHPs), nonclinical safety studies conducted in NHPs were useful in predicting potential human risks of IFNα therapy.

Monoclonal Antibodies

What are antibodies?

- An antibody is a protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target.
- The high specificity of antibodies makes them an excellent tool for detecting and quantifying a broad array of targets, from drugs to serum proteins to microorganisms.
- Within vitro assays, antibodies can be used to precipitate soluble antigens, agglutinate (clump) cells, opsonize and kill bacteria with the assistance of complement, and neutralize drugs, toxins, and viruses.

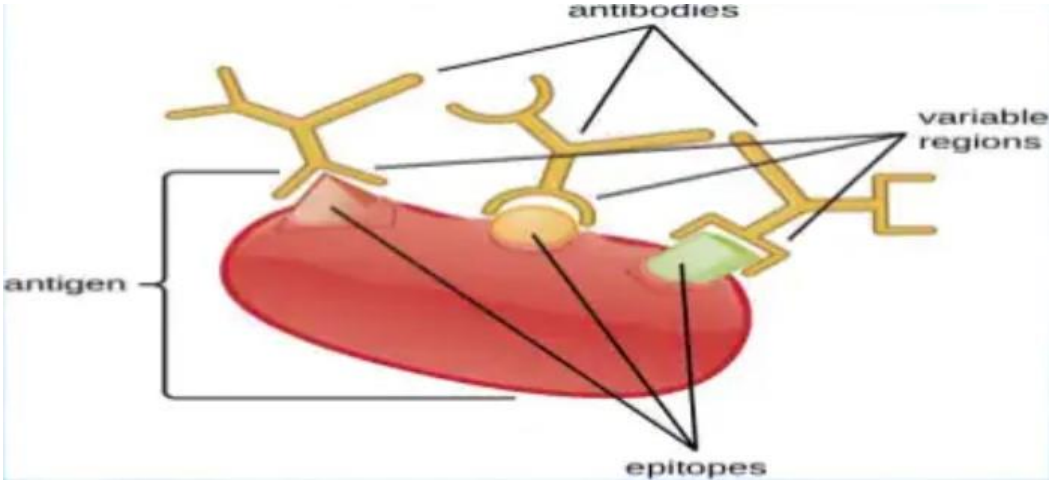

Figure 5: An antibody binds to a specific region on an antigen called an epitope. A single antigen can have multiple epitopes for different, specific antibodies.

An antibody binds to a specific region on an antigen called an epitope. A single antigen can have multiple epitopes for different, specific antibodies.

Monoclonal antibodies

- Monoclonal antibodies are identical immunoglobulins, generated from a single B-cell clone. These antibodies recognize unique epitopes, or binding sites, on a single antigen. Derivation from a single B-cell clone and subsequent targeting of a single epitope is what differentiates monoclonal antibodies from polyclonal antibodies.
- Polyclonal antibodies are antibodies that are derived from different cell lines. They differ in amino acid sequences.

Characters of monoclonal Antibodies

- Monoclonal antibodies (mAB) are single types of antibody that are identical and are directed against a specific epitope (antigen, antigenic determinant) and are produced by B-cell clones of a single parent or a single hybridoma cell line.
- A hybridoma cell line is formed by the fusion of one B-cell lymphocyte with a myeloma cell.
- Some myeloma cells synthesize single mAB antibodies naturally.

	Polyclonal antibodies	Monoclonal antibodies
Produced by:	Many B cell clones	A single B cell clone
Binds to:	Multible epitopes of all antigen used in the immunization	A single epitope of a single antigen
Antibody class:	A mixture of different Ab classes (isotypes)	All of a single Ab class
Ag-binding sites:	A mixture of Abs with different antigen-binding sites	All Abs have the same antigen binding sites
Potential for cross-reactivity:	High	Low

Table 5: Polyclonal and Monoclonal Antibodies

Advantages of using Monoclonal Antibodies:

- Though expensive, monoclonal antibodies are cheaper to develop than conventional drugs because it is based on tested technology
- Side effects can be treated and reduced by using mice-human hybrid cells or by using fractions of antibodies.
- They bind to specific diseased or damaged cells needing treatment.
- They treat a wide range of conditions.

Disadvantages of using Monoclonal Antibodies:

- Time-consuming project-anywhere between 6-9 months.
- Very expensive and needs considerable effort to produce them.
- Small peptides and fragment antigens may not be good antigens. monoclonal antibodies may not recognize the original antigen.
- Hybridoma culture may be subject to contamination.
- The system is only well developed for limited animals and not for other animals.
- More than 99% of the cells do not survive during the fusion process - reducing the range of useful antibodies that can be produced against an antigen ● It is the possibility of generating immunogenicity.

Monoclonal Antibodies: History and Development

- Paul Enrlich at the beginning of the 20th century coined the term "magic bullets" and postulated that, if a compound could be made that selectively targets a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity.
- In the 1970s, the B-cell cancer multiple myeloma was known. It was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein).

Figure 5.1: Paul Enrlich

- In 1975, Kohler and Milstein provided the most outstanding proof of the clonal selection theory by fusion of normal and malignant cells (Hybridoma technology) for which they received Nobel prize in 1984.

- In 1986, first monoclonal antibody was licenced by FDA. Orthoclone OKT_3 (muromonab-CD_3) which was approved for use in preventing kidney transplant rejection.

Figure 5.2: Georges J.F. Kohler and Cesar Milstein

Preparation of Monoclonal Antibodies

Monoclonal Antibody production or mAb is produced by cell lines or clones obtained from the immunized animals with the substances to be studied. Cell lines are produced by fusing B cells from the immunized animal with myeloma cells.

- To produce the desired mAB, the cells must be grown in either of two ways:
  by injection into the peritoneal cavity of a suitably prepared mouse (in vivo method) or by in vitro tissue culture.

- The vitro tissue culture is the method used when the cells are placed in the culture outside the mouse the mouse's body in the flask.

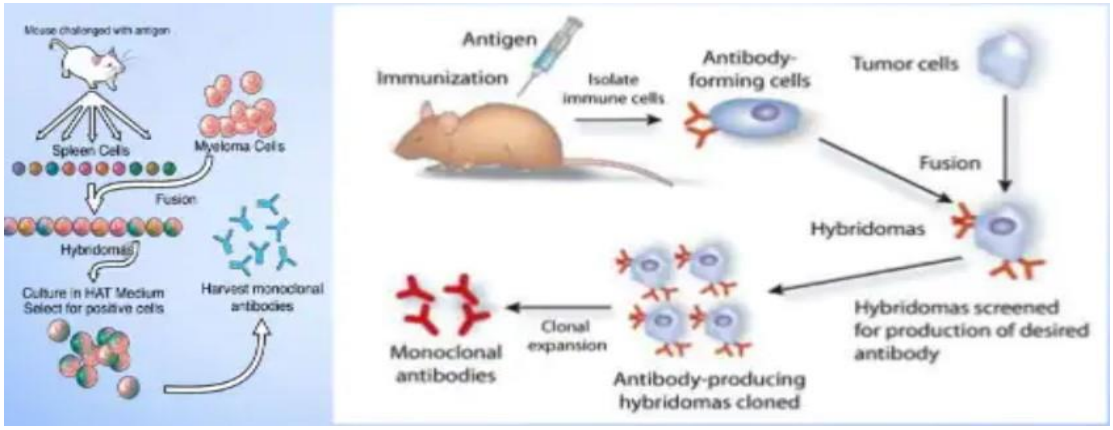

Figure 5.3: Preparation of Monoclonal Antibodies

Practical steps for production

- Immunize animal
- Isolate spleen cells (containing antibody-producing B cell)
- Fuse spleen cells with myeloma cell (using PEG)
- Allow unfused B cell to die
- Add aminopterin to culture and kill unfused myeloma cells
- Clone remaining cells (place 1 cell/well and allow each cell to grow into a cones of cell)
- Screen supernatant of each clone for the presence of the desired antibody ● Grow chosen clone of cells in tissue culture indefinitely ● Harvest antibody from the culture.
- 10.s 1000-2000 per mg

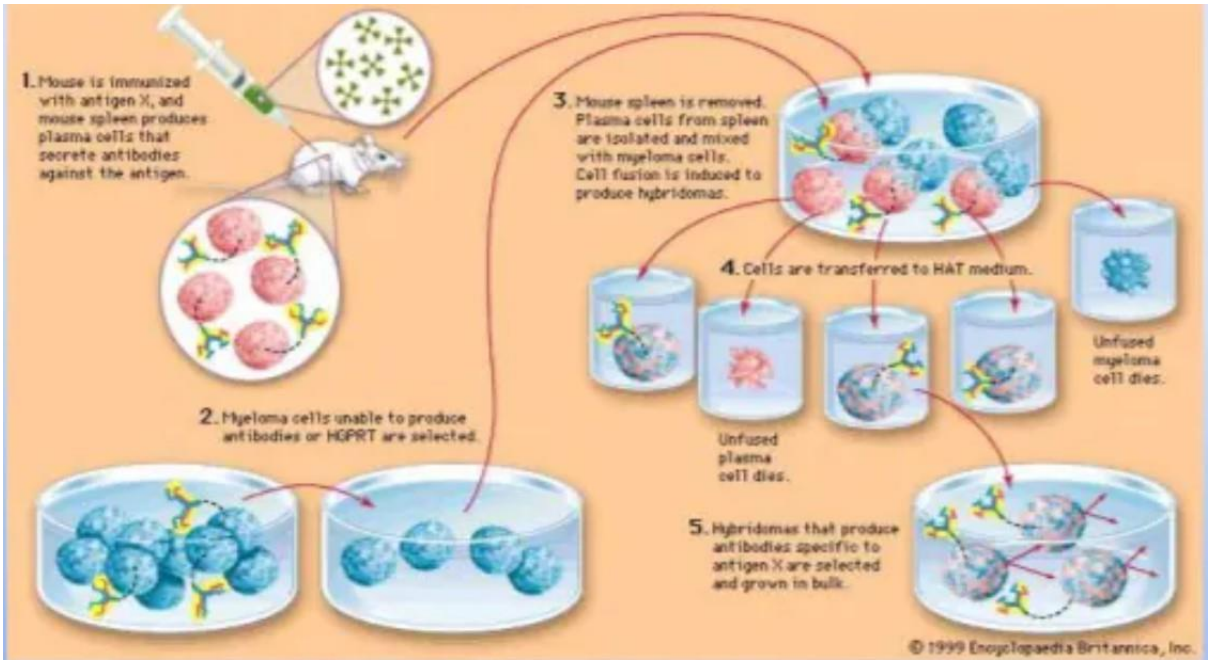

Figure 5.4: Practical Steps for Production

Evaluation

- Guidelines evaluation of monoclonal antibodies on biotherapeutic products", WHO, 1st March 2016
- Guideline on development, production, characterization, and specification for monoclonal antibodies and related products, EMA, 21t July 2016

1. Characterization of monoclonal antibodies

- Physicochemical characterization
- Immunological properties
- Biological activity
- Purity, impurity, and contaminants
- Quantity

1.1 Physicochemical characterization

- A physicochemical characterization program will generally include a determination of the class, subclass, light chain composition, and primary structure of the monoclonal antibody. The class or subclass of an antibody is defined by its heavy chain. There are five main classes of antibodies: M, G, A, E, and D. The method of antibody purification will differ based on the class.

- The amino acid sequence should be deduced from DNA sequencing and confirmed experimentally by appropriate methods (e.g. peptide mapping, amino acid sequencing, mass spectrometry analysis).
- The variability of N- and C- terminal amino-acid sequences should be analyzed (e.g. C-terminal lysine(s)).

Classes based on the constant region of heavy chains

- Immunoglobulin A (IgA)
- Immunoglobulin D (IgD)
- Immunoglobulin E (IgE)
- Immunoglobulin G (IgG)
- Immunoglobulin M (IgM)

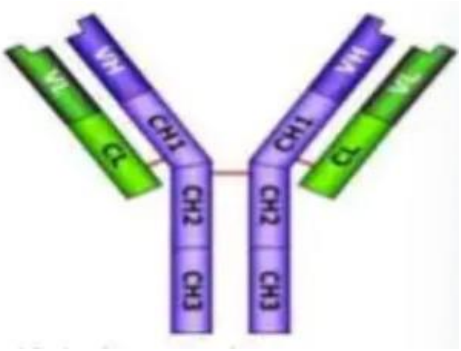

Figure 5.6: Immunoglobin light chain

Differentiation of heavy chains

- Length of C region, location of disulfide bonds, hinge region, distribution of carbohydrate

1.2 Immunological properties

- Binding assays of the antibody to purified antigens and defined regions of antigens should be performed, where feasible, to determine affinity, avidity, and immunoreactivity (including cross-reactivity with other structurally homologous proteins).
- Unintentional reactivity/cytotoxicity for human tissues distinct from the intended target should be documented. The epitope and molecule bearing the relevant epitope should be defined. This should include a biochemical identification of these structures (e.g. protein,

oligosaccharide, glycoprotein, glycolipid), and relevant characterization studies (amino acid sequence, carbohydrate structure) to the extent possible.

1.3 Biological activity

- The biological activity (i.e. the specific ability or capacity of a product to achieve a defined biological effect) should be assessed by appropriate in vitro assay(s).
- Where in vivo assays are necessary, the use of such assays should be thoroughly justified.
- The mechanism of action and the importance (or consequences) of the product effector functions with regards to the safety and efficacy of the product should be discussed.

1.4 Purity, impurity, and contaminants

- These methods generally include the determination of physicochemical properties such as molecular weight or size, extinction coefficient, electrophoretic profiles, chromatographic data, and spectroscopic profiles.
- Potential process-related impurities (e.g. HCP, host cell DNA, cell culture residues, downstream processing residues) should be identified, and evaluated qualitatively and/or quantitatively, using the chromatographic technique.
- Contaminants, which include all adventitiously introduced materials not intended to be part of the manufacturing process (e.g. microbial species, endotoxins) should be strictly avoided and/or suitably controlled.

1.5 Quality

- Quantity should be determined using a physicochemical and/or immunochemical assay.
- It should be demonstrated that the quantity values obtained are directly related to those derived using the biological assay.
- When this correlation exists, it may be appropriate to use a measurement of quantity rather than the measurement of biological activity in the product labeling and manufacturing processes, such as a filling.

2. Specifications

- Identity
- Purity and impurities
- Potency
- Quantity
- General tests
- Specifications are one part of a total control strategy designed to ensure product quality and consistency, and when tested, the product should comply with its specification.

Specifications should be set and take into account relevant quality attributes identified in characterization studies.

- The selection of tests to be included in the specifications is product-specific.
- The rationale used to establish the acceptable range of acceptance criteria should be described.

2.1 Identity

- The identity test(s) should be highly specific and should be based on unique aspects of the product's molecular structure and/or other specific properties (e.g. peptide map, anti-idiotype immunoassay, or another appropriate method).
- Considering the great similarity of the constant domains of different antibodies, more than one test (physicochemical, biological, and/or immunochemical) may be necessary to establish identity, and such test(s) should be able to discriminate other antibodies that may be manufactured in the same facility.

2.2 Purity and Impurities

- As noted in the characterization section, monoclonal antibodies may display a complex punty/impurity profile that should be assessed by a combination of orthogonal methods, and for which individual and/or collective acceptance criteria should be established for relevant product-related variants.
- For example, separation methods based on charge heterogeneity are considered to quantitatively and qualitatively monitor charge variants.
- Considering that glycosylation may have an impact on the pharmacokinetics of the product, and may modulate its immunogenic properties, appropriate acceptance criteria should be considered for this attribute. In addition, such control will further confirm the consistency of the product.

2.3 Potency

- Potency is the quantitative measure of biological activity based on an attribute of the product which is linked to the relevant biological properties. A relevant potency assay should be part of the specifications for drug substance and/or drug product, and should ideally reflect the biological activity in the clinical situation.
- For antibodies for which the clinical activity is only dependent on binding/neutralizing properties, a potency assay that measures binding to the target (.e. binding assay) is acceptable when appropriately justified.

2.4 Quantity

- The quantity of the drug substance, usually based on protein content (mass), is determined chromatographically using reference standards.

2.5 General tests

- Appearance, solubility, pH, osmolality, extractable volume, sterility, bacterial endotoxins, stabilizer, and water, is assessed where appropriate.

Major Applications:

(1) Diagnostic Applications

- Biochemical analysis
- Diagnostic Imaging

1a. Biochemical analysis

Routinely used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) in the laboratory.

These assays measure the circulating concentrations of hormones (insulin, human chorionic gonadotropin, growth hormone, progesterone, thyroxine, triodothyronine, thyroid stimulating hormone) and several other tissue and cell products (blood group antigens, blood clotting factors, interferon's, interleukins, tumor markers).

Eg. **Pregnancy** by detecting the urinary levels of human chorionic gonadotropin.

Hormonal disorders analysis of thyroxine, triiodothyronine.

Cancers estimation of plasma carcinoembryonic antigen in colorectal cancer, and prostate specific antigen for prostate cancer

1b. Diagnostic imaging

- Radiolabeled-MAbs are used in the diagnostic imaging of diseases, and this technique is referred to as immunoscintigraphy. The radioisotopes commonly used for labeling MAb are iodine 131 and technetium-99. The MAbtagged with radioisotope is injected intravenously into the patients.
- These MAbs localize at specific sites (say a tumor) which can be detected by imaging the radioactivity. In recent years, single-photon emission computed tomography (SPECT) cameras are used to give a more sensitive three-dimensional appearance of the spots localized by radiolabeled-MAbs.
- Myocardial infarction, DVT, atherosclerosis, etc.

(2) Therapeutic Applications

Direct use of MAbs as therapeutic agents ●
MAbs as targeting agents.

2a. Direct use of MAbs as therapeutic agents

- In destroying disease-causing organisms: MAbs promote efficient opsonization of pathogenic organisms (by coating with antibody) and enhance phagocytosis.
- In the immunosuppression of organ transplantation: In normal medical practice, immunosuppressive drugs such as cyclosporin and prednisone are administered to overcome the rejection of organ transplantation. In recent years, MAbs specific to T-lymphocyte surface antigens are being used for this purpose ●

In the treatment of cancer:

MAbs, against the antigens on the surface of cancer cells, are useful for the treatment of cancer. The antibodies bind to the cancer cells and destroy them via different pathways.

- In the treatment of AIDS: Genetic engineers have been successful to attach the Fc portion of mouse monoclonal antibody to human CD, molecule. This complex has a high affinity to bind to membrane glycoprotein gp120 of virus-infected cells. The Fc fragment induces cell-mediated destruction of HIV-infected cells.

2b. MAbs as targeting agents.

- The drugs can be coupled with MAb (directed against a cell surface antigen of the cells, say a tumor) and specifically targeted to reach the site of action.

Eg. Alkaline phosphatase for the conversion of phosphate pro-drugs.

- Carboxy peptidase for converting inactive carboxyl pro-drugs to active drugs.
- Lactamase for hydrolyzing ß-lactam ring containing antibiotics.

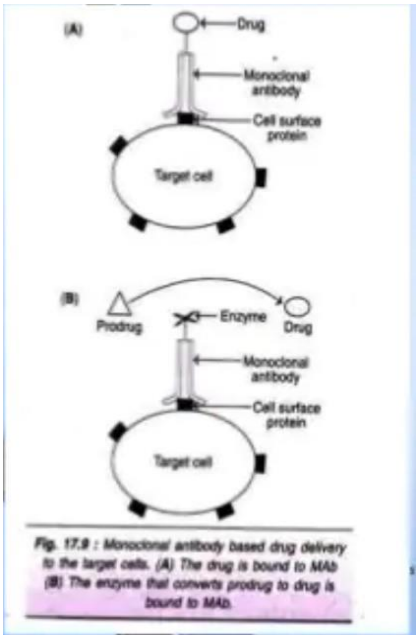

Figure 5.7: Monoclonal antibody based Drug

MAbs in the dissolution of blood clots:

- Fibrin is the major constituent of a blood clot that gets dissolved by plasmin. Plasmin in turn is formed by the activation of plasminogen by plasminogen activator.
- Tissue plasminogen activator (tPA) can be used as a therapeutic agent to remove blood clots.

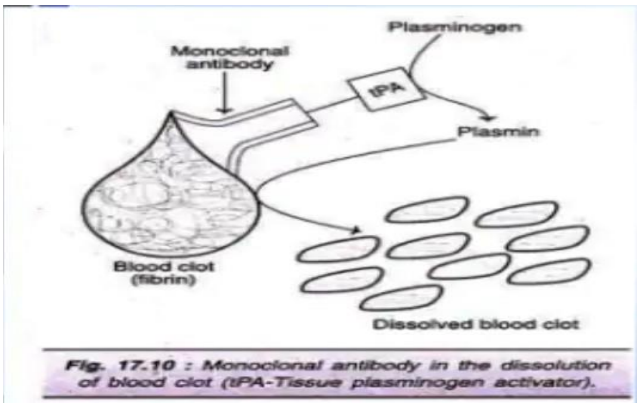

Figure 5.8: Monoclonal antibody in the dissolution of blood clot

Drug delivery through liposomes coupled to tissue-specific MAbs:

- Liposomes are sacs or vesicles formed spontaneously when certain lipid molecules are exposed to an aqueous environment.
- Drug entrapped in liposomes that are coated with MAbs directed against tissue-specific antigens is being tried for drug delivery.
- Unfortunately, the progress in this approach has been limited, since such liposomes do not reach the target cells.
- They are retained mostly in the liver and spleen (reticuloendothelial cells) and degraded.

3. Protein Purification

- Monoclonal antibodies can be produced for any protein. And the so produced MAb can be conveniently used for the purification of the protein against which it was raised. ● MAbs columns can be prepared by coupling them to cyanogen bromide activated Sepharose (chromatographic matrix). The immobilized MAbs in this manner is very useful for the purification of proteins by the immunoaffinity method.
- There are certain advantages of using MAbs for protein purification. These include the specificity of the MAb to bind to the desired protein, very efficient elution from the chromatographic column, and a high degree of purification.

Growth of Monoclonal Antibody Market

Figure 5.9: Growth of Monoclonal Antibody Market

A vast growing number of approved products in the market will increase the incidence of the monoclonal market in the coming future.

- About 75 monoclonal antibodies are currently approved by the FDA for use in humans for treating various diseases and conditions including cancer, chronic inflammatory diseases, transplantation, infectious diseases, and cardiovascular diseases.
- Glenmark, which is seeking permission from MHRA, U.K. for conducting Phase I clinical studies for one of its mAb candidates, GBR goo mAb targeting TrkA, the receptor of nerve growth factor to tackle chronic pain.
- India is fertile land for the mAb market due to Large patient base, growing economy, abundant manpower, and low R&D cost.

Few Commercially available mAb approved by FDA

Name	Trade name	Target	Use
Abciximab	ReoPro	CD41 (integrin alpha-IIb)	Platelet aggregation Inhibitor
Adalimubab	Humira	TNF-*alpha*	Rheumatoid arthritis, Crohn's Disease, Plaque psoriaris, psoriatic Arthritis
Alirocumab	Praluent	PCSK9	Hypercholesterolemia
Avilumab	Bavencio	PD-L1	Cancer
Benralizumab	Facenra	CD125	Asthma
Daclizumab	Zenapax	CD25	Organ transplant rejection
Daratumubab	Darzalex	CD-38	Multiple Myeloma

Table 5: Commercially available mAb approved by FDA

Immunotoxins

- An immunotoxin is a human-made protein that consists of a targeting portion linked to a toxin
- When the protein binds to that cell, it is taken in through endocytosis, and the toxin kills the cell.
- They are used for the treatment of some kinds of cancer and a few viral infections.
- An immunotoxin is a form of hybrid molecule blending materials from two different proteins which are not coupled
- Such combinations are said to be chimeric or fusion proteins reflecting the mix and material from different sources
- These chimeric proteins are usually made of a modified antibody or its fragment attached to a fragment of toxins
- The targetted portion is composed of the FV portion of an antibody that targets a specific cell type

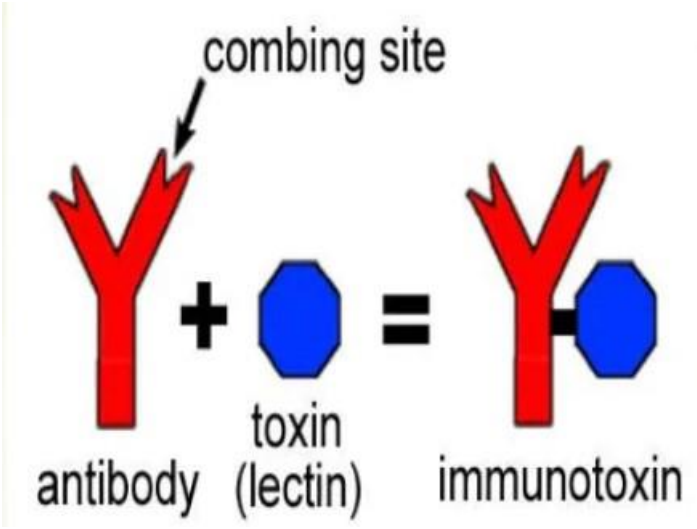

Figure 5.10: Immunotoxin

- The toxin is usually a cytotoxic protein from a bacteria or plant protein, from which the natural binding domains have been removed so that the FV directs the toxin to the antigen on the target cell.
- Sometimes, fusion proteins containing a toxin and a growth factor are also referred to as recombinant immunotoxins

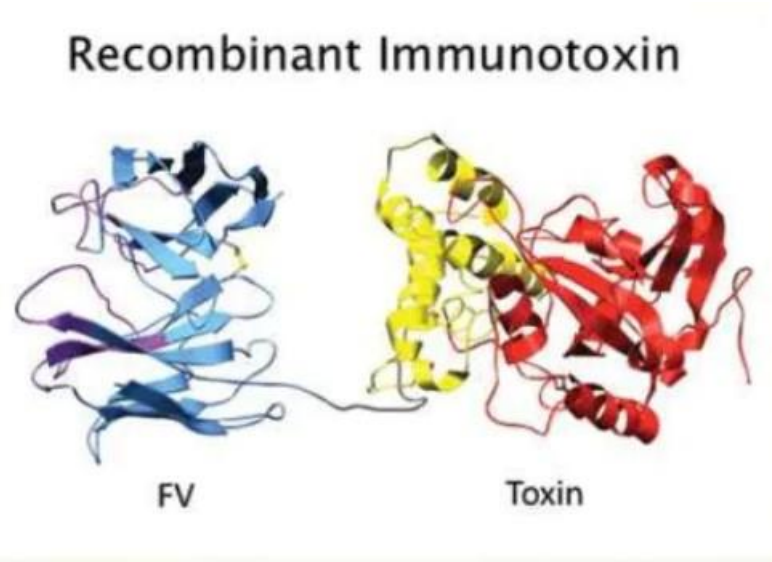

Figure 5.11: Recombinant Immunotoxin

- The immunotoxin bind to the surface antigen on a cancer cell enters the cell by endocytosis and kills it.
- Most protein immunotoxins are made from bacteria and plant toxins ● Toxins used to make immunotoxins have 3 domains:

  cell recognition domain which concentrates toxins on the surface of the target
  cell translocation domain: enables toxins to coss membrane to reach the cytosol
  death domain: inactivates vital cellular proteins and kills the cell

PRODUCTION OF IMMUNOTOXINS

Immunotoxins are produced in Escherichia coli transformed with a plasmid encoding the recombinant toxin.

A common method of producing material for clinical trials is harvesting recombinant protein from insoluble bacterial inclusion bodies

The insoluble protein can be washed extensively with detergent to remove endotoxin, solubilized, denatured, and reduced in guanidine-dithioerythritol solution.

The recombinant protein is then renatured by rapid dilution into refolding redox buffer containing arginine and glutathione, and the dialyzed renatured protein purified by anion exchange and sizing chromatography.

The other method is that harvesting the protein from the cytoplasm or cell lysate and then using an affinity column to capture the dilute protein.

IMMUNOTOXIN MECHANISM

The mechanism by which immunotoxins work to kill diseased cells in the body is quite simple.

Using AIDS therapy as an example, let's say we have developed an immunotoxin to kill HIV-infected cells by raising antibodies that bind to GP120, a viral protein found on the outside of only HIV-infected cells.

Once an AIDS patient has been treated, the immunotoxin floats around in the bloodstream until it binds to a GP120 molecule on the outside of an infected cell.

Once bound, the GP120-immunotoxin complex gets pulled inside the cell by endocytosis, where it is either localized to an acidified endosome (if DT is the toxin), or the endoplasmic reticulum (ER) and trans-Golgi apparatus in the cell.

Inside these organelles, the linker holding the toxin to the antibody is cleaved.

Usually, the linker is made with an internal disulfide bond, so that it is stable in the oxidizing atmosphere outside the cell and cleaved by a reduction in the reducing environment inside the cell.

Once freed from the antibody, the toxin now catalytically inactivates the protein synthesis machinery of the cell.

The bacterial toxins perform this by inactivating the ribosome accessory protein elongation factor-2 (EF-2).

The plant RIPs accomplish their task by cleaving a single adenine base from the ribosomal RNA so that it can no longer bind EF-2.

Either way, the inactivation of protein synthesis leads to the death of the cell.

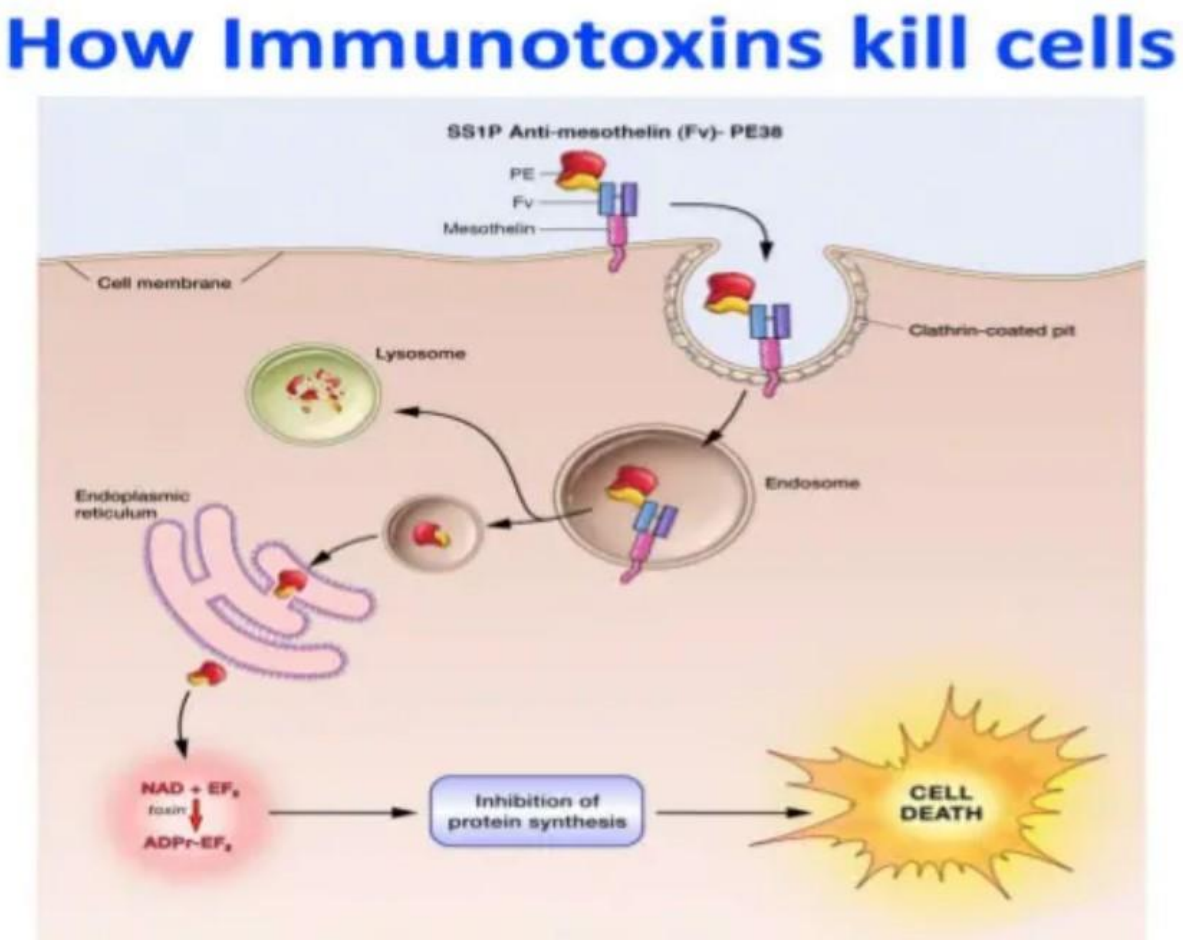

Figure 5.12: Moxetumomab pasudotox (Moxe) binds CD22 receptors on the surface of cancerous B cells, where it is internalized and processed to release its toxic payload.

DEVELOPMENT OF IMMUNOTOXINS

The first-generation IT is produced chemically by coupling native toxins to Ab using crosslinking reagents that form disulfide bonds connecting the toxins to Ab.

The second-generation immunotoxins are made by the coupling method. The drawback was heterogeneity and vascular leak syndrome

The third IT was made from recombinant DNA technique and combine various fragments of an Ab and toxins without their cell-binding domains on the same proteins.

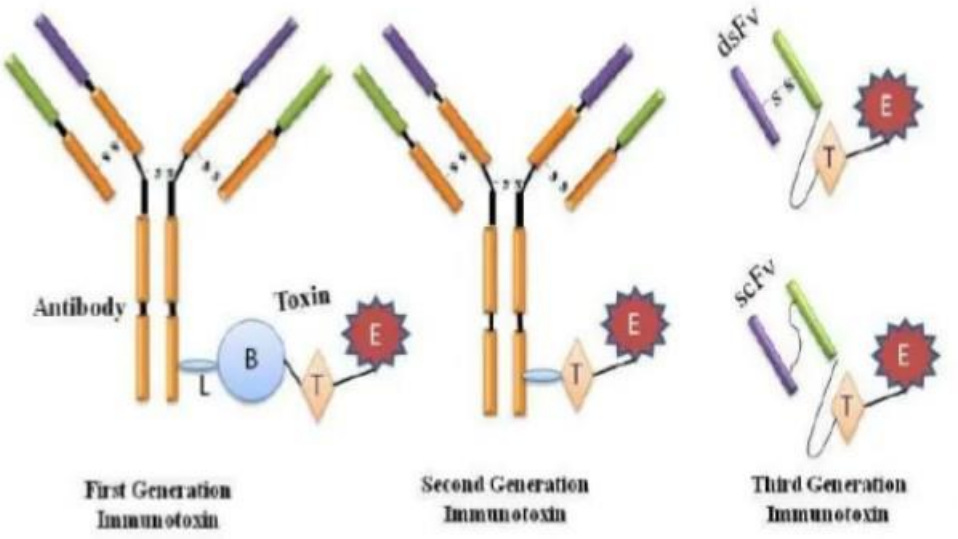

Figure 5.13: Structural representation of the three generations of immunotoxins. L- chemical linker; B- binding domain of toxin; T- translocational domain of toxin; E- enzymatic domain of toxin; dsFv- double strand of variable of antibody; scFv- single chain variable of fragment of antibody.

TARGETS OF IMMUNOTOXINS

Immunotoxins in the Treatment of Hairy Cell Leukemia:

Hairy Cell Leukemia, (HCL) is a rarely seen cancer of cells known as B-lymphocytes.

The new experimental therapy involves the injection of an immunotoxin designed to destroy the cancerous cells.

This immunotoxin, known as LMB-2, is made by using recombinant DNA technology to attach part of an antibody molecule (designed to recognize a substance called CD25) to the toxin produced by bacteria called Pseudomonas,

Immunotoxins in the Treatment of Acute Myelogenous Leukemia:

An immunotoxin is developed as a conjugate of a monoclonal antibody that binds CD33, a cell-surface molecule expressed by the cancerous cells in acute myelogenous leukemia (AML)

Commercially this immunotoxin is named Mylotarg.

Mylotarg is the first immunotoxin to show promise in the fight against cancer.

Immunotoxins in the Treatment of Lymphomas:

A conjugate of monoclonal antibody against the CD_{22}, a molecule found on the surface of some leukemias and lymphomas with pseudomonas exotoxin, a bacterial product that blocks protein synthesis in cells is developed.

Commercially this immunotoxin has been named BL_{22}.

Immunotoxins in the Treatment of Solid Tumour:

Targeting solid tumors with immunotoxins is much more difficult than targeting hematologic tumors.

Not only are the cellular junctions tighter and the tumor cells more tightly packed, but the patients are less immunosuppressed and more likely to make neutralizing antibodies to the toxin.

The monoclonal antibody 8H9 was produced by immunizing human neuroblastoma cells to mice.

8H9 was highly reactive against human brain tumors, childhood sarcomas, and neuroblastomas.

The characterization of 8H9 and its antigen presented on the surface of cancer cells suggests that 8H9 may be useful for targeted cancer therapy.

EX-Vivo Gene Therapy

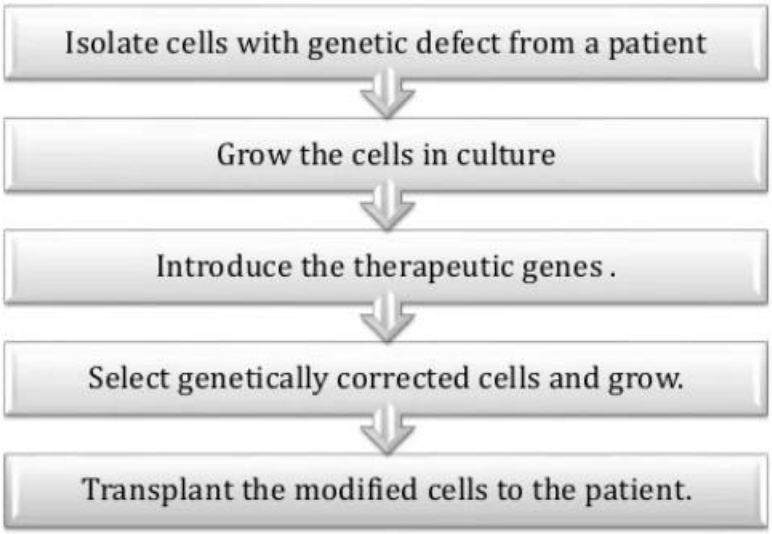

Figure 5.14: EX-Vivo Gene Therapy

EXAMPLE OF EX VIVO GENE THERAPY

1st gene therapy - to correct the deficiency of enzyme, Adenosine deaminase (ADA).
Performed on a 4yr old girl Ashanthi DeSilva.
Was suffering from SCID- Severe Combined Immunodeficiency.
Caused due to defect in the gene coding for ADA.
Deoxy adenosine accumulates and destroys T lymphocytes.
Disrupts immunity, suffers from infectious diseases, and dies at a young age.\

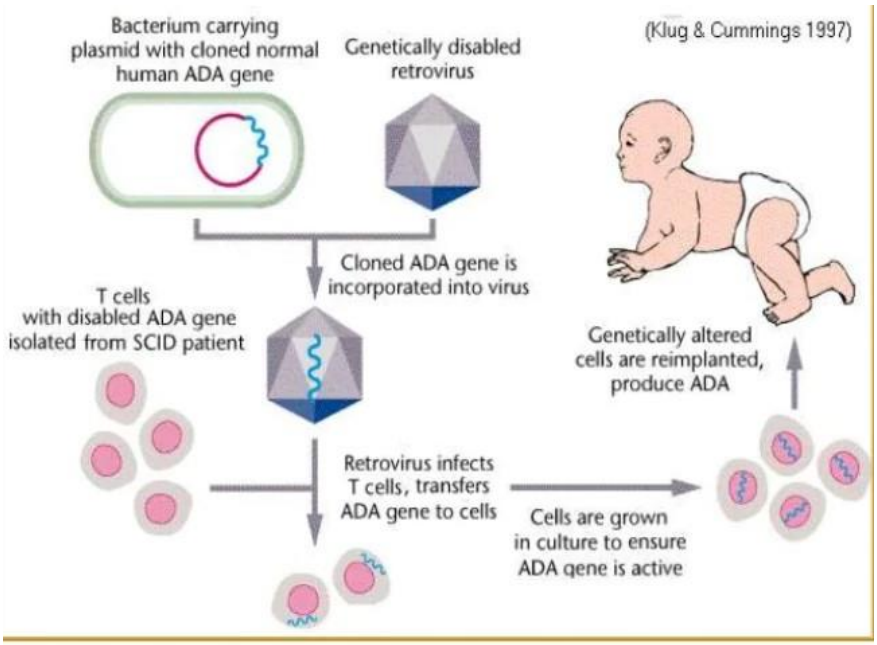

Figure 5.15: EX VIVO GENE THERAPY

IN VIVO GENE THERAPY

Direct delivery of the therapeutic gene into the target cell into the patient's body.
Carried out by viral or non-viral vector systems.
It can be the only possible option in patients where individual cells cannot be cultured in vitro in sufficient numbers (e.g. brain cells).
In vivo gene transfer is necessary when cultured cells cannot be re-implanted in patients effectively.

EXAMPLE OF IN VIVO GENE THERAPY

-Therapy for cystic fibrosis

In patients with cystic fibrosis, a protein called cystic fibrosis transmembrane regulator (CFTR) is absent due to a gene defect.

In the absence of CFTR chloride ions concentrate within the cells and draw water from the surrounding.

This leads to the accumulation of sticky mucous in the respiratory tract and lungs.

Treated by in vivo replacement of defective gene by adenovirus vector.

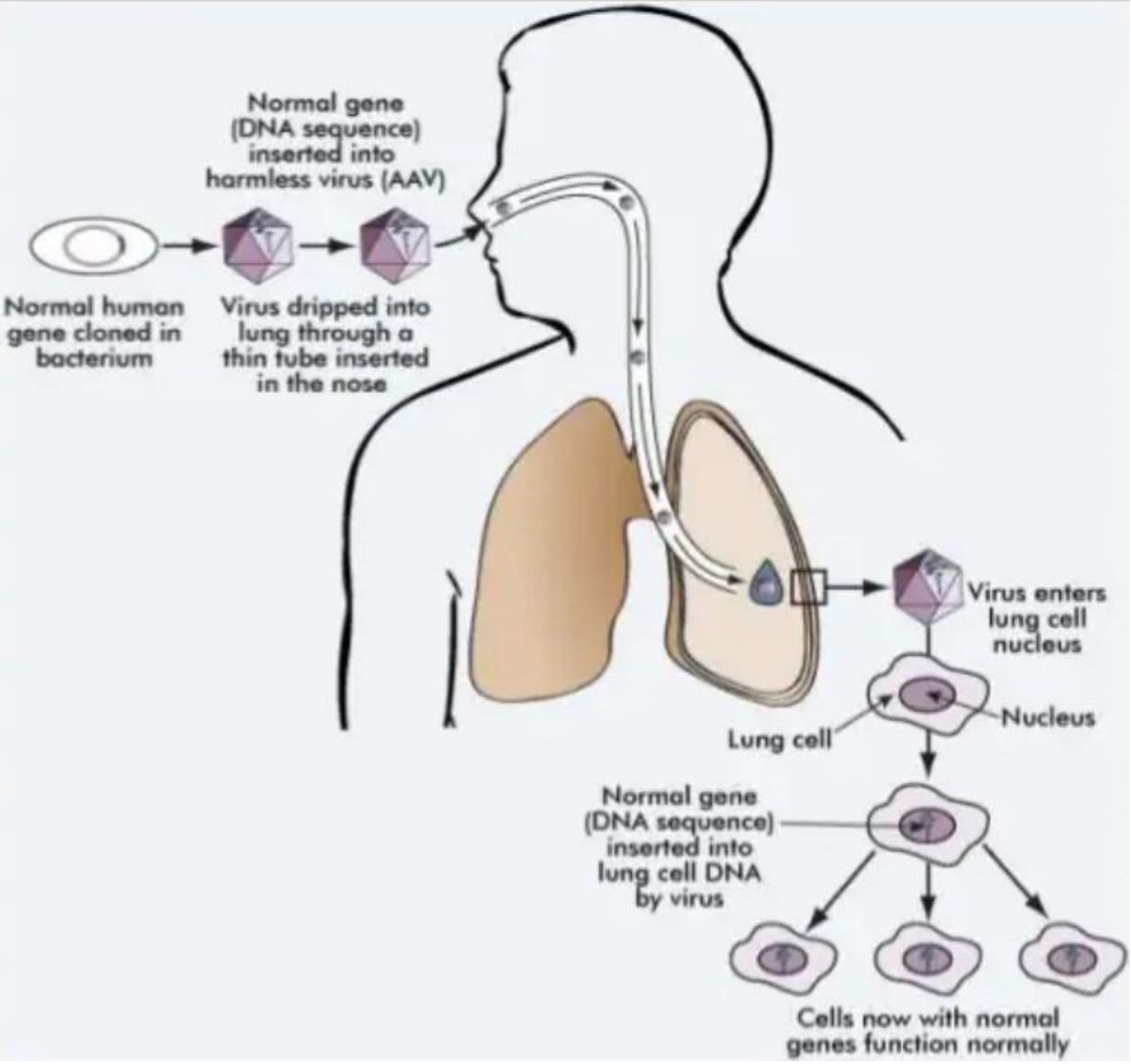

Figure 5.16: IN VIVO GENE THERAPY

Production of vaccine

Vaccines are produced on a large scale as they need to be administrated to large populations of children and adults to be effective as a public health tool.

The production of vaccines has several stages through which vaccines are produced.

Stages of vaccine production

- ✔ Generation/Isolation of Antigen
- ✔ Purification
- ✔ Inactivation
- ✔ Formulation
- ✔ Filling
- ✔ Batch release
- ✔ Packing and shipping

Figure 5.17: Several stages through which vaccines are produced

01. Generation/Isolation of antigen

- ✔ Viruses are grown on primary cells such as cells from chicken embryos or using fertilized eggs (e. g. influenza vaccine)or cell lines that reproduce repeatedly(e.g. hepatitis A).
- ✔ Bacteria are grown in bioreactors which are devices that use a particular growth medium that optimizes the production of the antigens
- ✔ Recombinant proteins derived from the pathogen can be generated either in yeast, bacteria, or cell cultures.
- ✔ Production starts by growing viruses bacteria in the lab a wide range of program meter manages like
    - Temperatures
    - PH
    - Oxygen Rate Sterility
    - Homogeneity

02. Purification

- The microorganisms are extracted from the environment through a process called purification which are ruminated any traces in culture media.

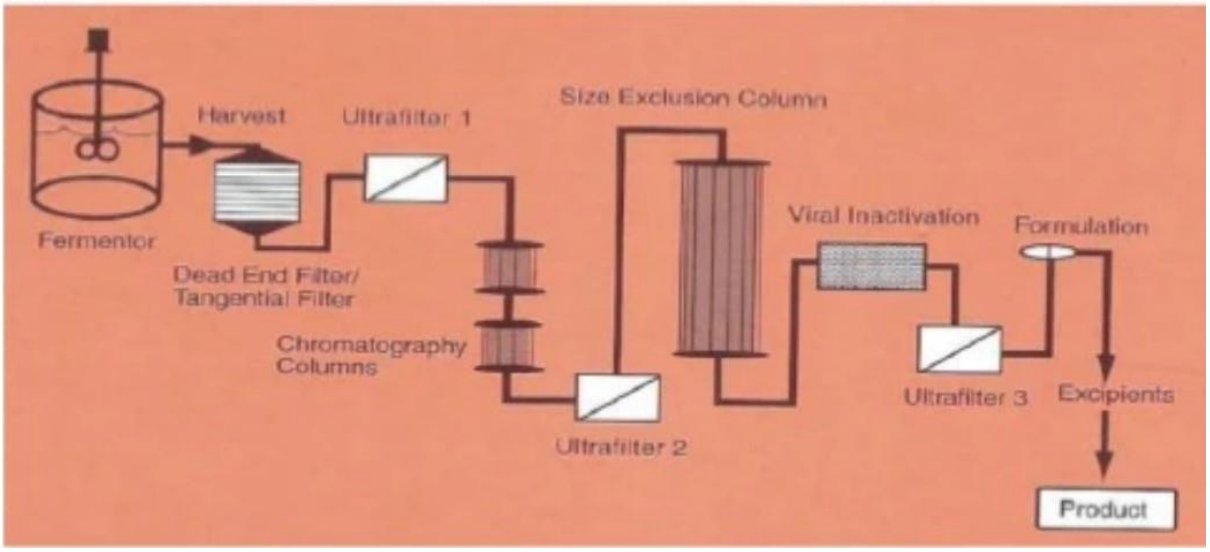

Figure 5.18: Steps for vaccine Production

03.Inactivation

- Then inactivate the virus or Bacteria that ruminates to the ability to cause disease.
- We retain its ability to leisure precautionary immune response from the body.
- That activated virus or bacteria is called valence.

04. Formulation

- Once the antigen is developed the vaccine is formulated by adding adjuvant, stabilizers, and preservatives.
- The role of the adjuvant is to enhance the immune response of the antigen.
- The stabilizers increase the storage life, and preservatives use allow multi-dose vials.

05. Filling

- When it's time for the vaccine to be administrated it would be reconstituted by combining the powder with diluents.
- The vaccine is liquid or freeze-dried form and diluents are needed and then filled into seeds or syringes.
- Each phase undergoes visual inspection. Quality containers are scrutinized by the human eye and sterile in digital surveillance technology.

06. Batch release

- Samples are taken from each batch.
- After vaccines are successfully passed both sides of the test distribution began.
- No exception if a batch is not matched with quality criteria will be destroyed.

07. Packing and shipping

- For the packing and shipping vaccine must be stored in 2º to 8ºC.
- Temperatures tracking device is included in each shipment.

Significance of vaccine production

- Makes good economic sense, and meets the need to care for the weakest members of societies. Reducing global child mortality.
- Global life-saving immunization against deadly diseases like meales, Hib, and rotavirus.

MOLECULAR FARMING

What is Molecular Farming?

- The use of whole organisms, organs, tissues or cells, or cell cultures, as bioreactors for the production of commercially valuable products via recombinant DNA techniques.
- Molecular farming is an application of GM Technology.
- It is also known as biopharming or gene pharming.
- Molecular pharming" is a technology that uses plants to produce large quantities of pharmaceutical substances such as vaccines and antibodies.
- Plant molecular farming is the production of pharmaceutically important and commercially valuable diagnostic proteins and/or industrial enzymes in plants. Combination of biotechnology and agriculture to produce new biomolecules for the benefit of a human being.
- Molecular farming started about 20 years ago with the promise to produce therapeutic molecules.
- Some therapeutic molecules are very expensive to produce.
- Falls under the category of green biotechnology.
- use of bacteria to assure better crop yields instead of pesticides and herbicides
- production of superior plants by stimulating the early development of their root systems
- use of plants to remove heavy metals such as lead, nickel, or silver, which can then be extracted ("mined") from the plants
- genetic manipulation to allow plant strains to be frost-resistant
- use of genes from soil bacteria to genetically alter plants to promote tolerance to fungal pathogens
- use of bacteria to get plants to grow faster, resist frost, and ripen earlier.

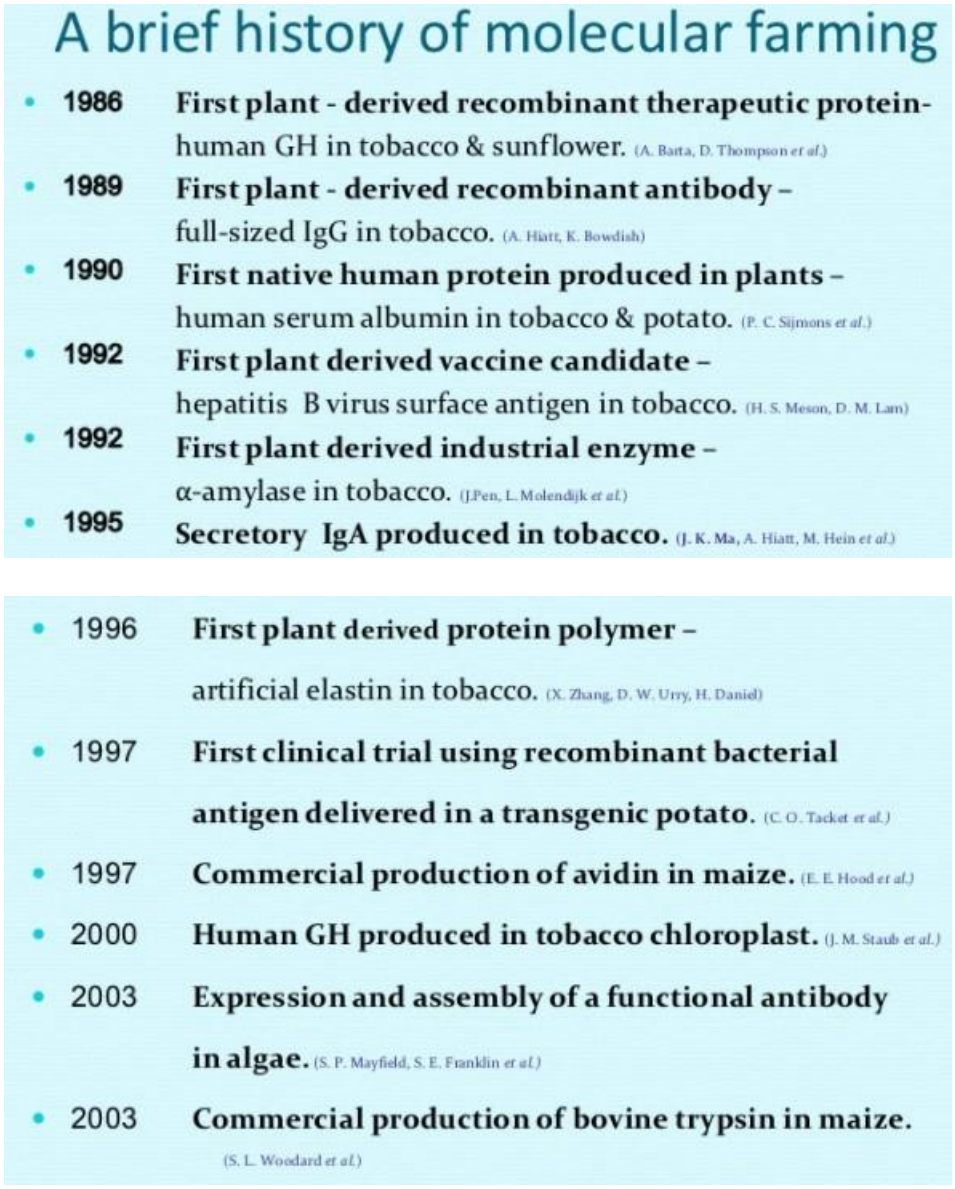

Figure 5.19: History of Molecular Farming

Molecular Farming Strategy

- Clone a gene of interest
- Transform the host platform species
- Grow the host species, recover biomass
- Process biomass
- Purify product of interest
- Deliver product of interest

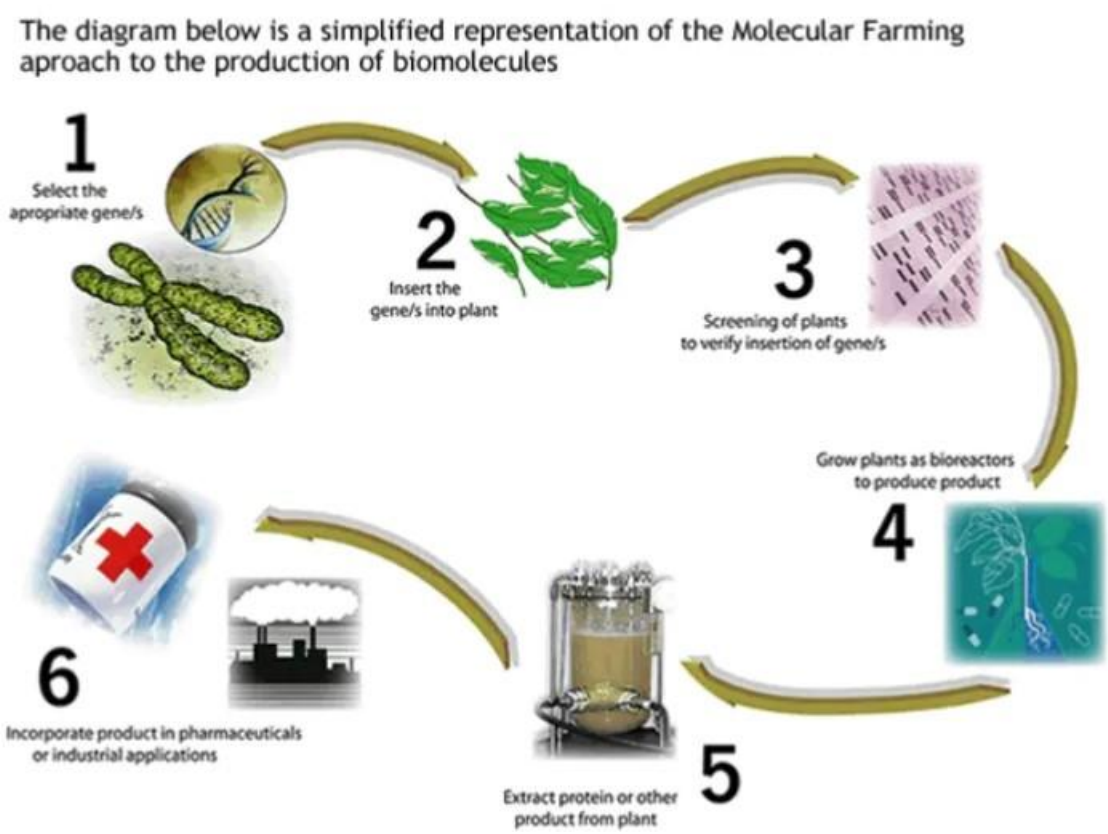

Figure 5.20: Molecular Farming approach to the production of biomolecules

Molecular Farming Hosts

- Bacteria
- Yeasts, (single-celled fungi)
- Unicellular algae
- Mammalian, insect, plant, and filamentous fungal cell cultures
- Whole plants, (corn, barley, rice, duckweed, moss protonema)
- Whole animals, (insects, birds, fish, mammals) Bacteria :

- Do not produce glycosylated full-sized antibodies.
- Contaminating endotoxin is difficult to remove.
- Recombinant proteins often form inclusion bodies.
- Labour- and cost-intensive refolding in vitro necessary.
- Lower scalability
- Preferred for the production of small, aglycosylated proteins like Insulin, interferon-β.

Animal Based Systems:

- Limited by legal and ethical restriction
- Require expensive equipment & media
- Delicate nature of mammalian cells
- Human pathogens and oncogenes
- Scaling up problems

Plant Molecular Farming

- Significantly lower production cost than with transgenic animals, fermentation or bioreactors.
- Infrastructure & expertise already exists for the planting, harvesting & processing of plant material.
- Plants contain no known human pathogens (such as prions, virions, etc.) that could contaminate the final product.
- Higher plants generally synthesize proteins from eukaryotes with correct folding, glycosylation & activity.
- Plant cells can direct proteins to environments that reduce degradation and therefore increase stability.
- Low ethical concerns.
- Easier purification (homologs don't pose any purification challenge, e.g. serum proteins or antibodies).
- Versatile (production of a broad diversity of proteins).
- Take more time to develop
- Transgene & protein pollution

Animal Biosafety Level 1

Animal Biosafety Level 1 is suitable for work involving well characterized agents that are not known to cause disease in immunocompetent adult humans, and present minimal potential hazard to personnel and the environment. ABSL-1 facilities should be separated from the general traffic patterns of the building and restricted as appropriate. Special containment equipment or facility design may be required as determined by appropriate risk assessment. Personnel must have specific training in animal facility procedures and must be supervised by an individual with adequate knowledge of potential hazards and experimental animal procedures.

Animal Biosafety Level 2

Animal Biosafety Level 2 builds upon the practices, procedures, containment equipment, and facility requirements of ABSL-1. ABSL-2 is suitable for work involving laboratory animals infected with agents associated with human disease and pose moderate hazards to personnel and the environment. It also addresses hazards from ingestion as well as from percutaneous and mucous membrane exposure.

ABSL-2 requires that:

1. Access to the animal facility is restricted;
2. Personnel must have specific training in animal facility procedures, the handling of infected animals and the manipulation of pathogenic agents;
3. Personnel must be supervised by individuals with adequate knowledge of potential hazards, microbiological agents, animal manipulations and husbandry procedures; and
4. Procedures involving the manipulation of infectious materials, or where aerosols or splashes may be created, should be conducted in BSCs or by use of other physical containment equipment. Appropriate personal protective equipment must be utilized to reduce exposure to infectious agents, animals, and contaminated equipment. Implementation of employee occupational health programs should be considered.

Animal Biosafety Level 3

Animal Biosafety Level 3 involves practices suitable for work with laboratory animals infected with indigenous or exotic agents, agents that present a potential for aerosol transmission and agents causing serious or potentially lethal disease. ABSL-3 builds upon the standard practices, procedures, containment equipment, and facility requirements of ABSL-2. ABSL-3 laboratory has special engineering and design features.

ABSL-3 requires that:

1. Access to the animal facility is restricted;
2. Personnel must have specific training in animal facility procedures, the handling of infected animals and the manipulation of potentially lethal agents;
3. Personnel must be supervised by individuals with adequate knowledge of potential hazards, microbiological agents, animal manipulations and husbandry procedures; and
4. Procedures involving the manipulation of infectious materials, or where aerosols or splashes may be created, must be conducted in BSCs or by use of other physical containment equipment. Appropriate personal protective equipment must be utilized to reduce exposure to infectious agents, animals, and contaminated equipment. Employee occupational health programs must be implemented.

Animal Biosafety Level 4

Animal Biosafety Level 4 is required for work with animals infected with dangerous and exotic agents that pose a high individual risk of life-threatening disease, aerosol transmission, or related agent with unknown risk of transmission. Agents with a close or identical antigenic relationship to agents requiring ABSL-4 containment must be handled at this level until sufficient data are obtained either to confirm continued work at

this level, or to re-designate the level. Animal care staff must have specific and thorough training in handling extremely hazardous, infectious agents and infected animals. Animal care staff must understand the primary and secondary containment functions of standard and special practices, containment equipment, and laboratory design characteristics. All animal care staff and supervisors must be competent in handling animals, agents and procedures requiring ABSL-4 containment. Access to the animal facility within the ABSL-4 laboratory is controlled by the animal facility director and/or laboratory supervisor in accordance with institutional policies.

There are two models for ABSL-4 laboratories:

1. A *Cabinet Laboratory* where all handling of agents, infected animals and housing of infected animals must be performed in Class III BSCs.
2. A *Suit Laboratory* where personnel must wear a positive pressure protective suit (See Appendix A); infected animals must be housed in ventilated enclosures with inward directional airflow and HEPA filtered exhaust; infected animals should be handled within a primary barrier system, such as a Class II BSC or other equivalent containment system.

ABSL-4 builds upon the standard practices, procedures, containment equipment, and facility requirements of ABSL-3. However, ABSL-4 cabinet and suit laboratories have special engineering and design features to prevent microorganisms from being disseminated into the environment and personnel. The ABSL-4 cabinet laboratory is distinctly different from an ABSL-3 laboratory containing a Class III BSC.

BIO-SAFETY LEVEL

- BSL-1 This level is suitable for work involving well-characterized agents not known to consistently cause disease in healthy adult humans, and of the minimal potential hazard to laboratory personnel and the environment
- BSL-2 This level is similar to Biosafety Level 1 and is suitable for work involving agents of the moderate potential hazard to personnel and the environment.
- BSL-3 This level deals with work which is done with indigenous or exotic agents which may cause serious or potentially lethal disease after inhalation
- BSL-4 This level is required for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections, agents which cause severe to fatal disease in humans for which vaccines or other treatments are not available

BIO-SAFETY LEVEL 1 AND 2

The international biohazard warning symbol and sign must be displayed on the doors of the rooms where microorganisms of Risk Group 2 or higher risk groups are handled.

Figure 5.21: Biohazard warning symbol

Personal protection

- Laboratory coveralls, gowns, or uniforms must be worn at all times for work in the laboratory.
- Appropriate gloves must be worn for all procedures.
- After use, gloves should be removed aseptically and hands must then be washed.
- Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas

- ❖ Safety glasses, face shields, or other protective devices must be worn when it is necessary to protect the eyes and face from splashes, impacting objects, and sources of artificial ultraviolet radiation.
- ❖ It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in canteens, coffee rooms, offices, libraries, staff rooms, and toilets.
- ❖ Eating, drinking, smoking, applying cosmetics, and handling contact lenses are prohibited in the laboratory working areas.

Transgenic animals are produced for four basic reasons: to improve animal health, to increase productivity and improve product quality, to mitigate the environmental impact of food animal production, and to produce therapeutics. To date, scientists have been able to add, delete, silence, or partially activate genes of interest. To regulate such a powerful technology predicated on limited background information is a challenge not only to the regulators but also to the developers who strive to prove that these animals are safe by demonstrating bioequivalency to their conventional counterparts. The regulations are based on the principle of substantial equivalence from the point of view of human food safety, and data is required to elucidate molecular characterization, nutritional similarities, and toxicological studies to substantiate that the animal product is safe. To address the concerns connected with the environmental release of transgenic animals, the regulatory framework should take into account the ability of genetically modified animals to survive and compete with conventional populations. They should consider biosafety issues to prevent adverse effects of genetic modification on bio-diversity and to prove that the animals have no negative environmental impact. The challenge to regulate the animal health component of transgenic animals is addressed by animal welfare considerations and risk assessments, to ensure that such animals are not susceptible to diseases or act as vectors for disease-promoting organisms under their transgenic origin. The Canadian regulatory system relies on the "precautionary principle" in its approach to regulate the "product" instead of the "process". The regulatory framework captures transgenic animals under a 'safety net' created by the Canadian Environmental Protection Act (CEPA), administered by Environment Canada. The New Substances Notification Regulations (NSNR) (http://www2.ec.gc.ca/substances/nsb/enq/qui_e.htm) of this Act define the standards and guidelines for the assessment of transgenic animals before their introduction into the environment. Assessment must be based on the intended use, whether it is food, biopharmaceutical, or organ transplantation. Food from transgenic animals is assessed for safety by Health Canada under its Novel Foods Regulations of the Food and Drugs Act. Transgenic animals developed for food purposes are considered novel foods if (i) they have no previous history of safe use; (ii) the manufacturing process has never been previously used, and (iii) food derived from plants/animals/ organisms that have been genetically modified falls outside the expected standard range. To date, no transgenic animal has been approved for food use or release in Canada. The link between animal feed and the human food chain necessitates establishing strict criteria for the evaluation of feeds containing genetically modified materials. The regulatory issues related to feeding assessments tend to overlap the assessment standards for novel foods and the environmental assessment of

genetically modified organisms. Feed containing any genetically modified organism is considered Novel Feed, defined as a feed comprising an organism or organisms (i.e. from plant, microbial or animal source) or parts or products thereof that is not set out in schedules or has a novel trait. Novel traits include characteristics that have been intentionally selected, created, or introduced through a genetic change or based on the valid scientific rationale that is not substantially equivalent in terms of their use and safety from environmental, human, and animal health points of view. The Canadian Food Inspection Agency (CFIA) administers the national livestock feed program under the authority of the Feeds Act and Regulations, to verify that livestock feed manufactured and sold in Canada is safe, effective, and labeled properly. The regulation of veterinary biologics, to prevent and diagnose infectious diseases in animals, relies on effective science-based regulatory controls that take into account safety, efficacy, and availability. To meet the licensing requirements, veterinary biologics must be shown to be pure, potent, safe, and effective when used according to label recommendations. The detailed description of the process followed in preparing a veterinary biologic, tests used to establish its efficacy, purity, and potency, and the methods employed in handling, storing, and labeling a veterinary biologic are critical to effective regulation. Veterinary biologics are regulated by the CFIA under the Health of Animals Act and Regulations. The aspects of permit issuance and the bio-safety protocols followed in the production and testing of biologics are critically assessed before products are approved for use. The Canadian system of regulation for feeds, veterinary biologics, and transgenic animals may apply to other countries in the process of establishing a regulatory framework for biotechnology-derived products. An effective regulatory filter can permit safe products while forming a secure barrier for those that pose an unacceptable risk. However, even though extrapolation of regulatory principles from the Canadian system may be adaptable in developing countries, there remains a wide scope for improvements, just as the Canadian system is undergoing further development. The fast pace at which methodological advancements are currently being introduced indicates that the regulatory system will have to be constantly reviewed, altered, and improved in a manner that keeps up with the technological leaps to address the public concern over transgenesis in agriculture.

Rules for the Manufacture, Use / Import / Export, and Storage of Hazardous Microorganisms, Genetically Engineered Organisms or Cells (Rules 1989)

- Notified in the exercise of powers under sections 6,8 & 25 of the EPA, 1986 on 5th Dec 89.
- Rules come into force from 01.10.1993.

Application of Rules:

- Manufacture, import, and storage of microorganisms and Gene-technological products.
- Genetically engineered organisms, microorganisms, and cells and correspondingly to any substances and products and food pieces of stuff, etc.

- Sale, any kind of handling, exportation, importation, production, manufacture, processing, storage, drawing off, packaging, repackaging of GMOs and drugs & pharmaceuticals, foodstuffs, etc. from GMOs and Gene technology products.

State Biotechnology Co-Ordination Committee (SBCC)

Main functions

- Powers to inspect, investigate and to take punitive action in case of violations of statutory provisions through the State Pollution Control Board or the Directorate of Health etc.
- To review periodically the safety and control measures in various institutions handling GMOs.
- To act as nodal agency at State level to assess the damage, if any, due to release of GMOs and to take on site control measures.