Post-Graduate Degree Programme
(CBCS)
in
ZOOLOGY
(M.Sc. Programme)

SEMESTER-III

Cell and Development Biology

ZDSE(MJ)T-301

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
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Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2020 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Manas Kumar Sanyal, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

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Their persistent and coordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

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Theory (Discipline Specific Elective – Major I) - [ZDSE(MJ)T-301]

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	IV	Chemical composition and properties of fluorescence dye, principle and application: DAPI, Propidium Iodide, Acridine orange, Rhodamine, DCFDA, Hoechst		
	V	Study of subcellular organelle under light microscopy and electron microscopy.		
	VI	Radio labeling techniques: Detection and measurement of different types of radioisotopes normally used in biology, incorporation of radioisotopes in biological tissues and cells		
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	Total counseling session 12hrs.			

UNIT I

Fixation, staining and application: Solutions: Definition, Composition, Expression, Ideal & non-ideal Solution

Objective: In this unit, you will learn about Solutions; its Definition, Composition, Expression, Ideal & non-ideal Solution.

i. <u>Solution</u>: A true solution is made up of at least two components, the dispersed (the solute) and the dispersion (the solvent).

The solute does not settle down and remains evenly dispersed. Its particles are 1 nm or less in size and occur in molecular form. A spoonful of common salt or sucrose when stirred in a beaker full of water gets dissolved to produce a clear solution. Two components are obvious in this experiment; the common salt or sucrose (solute) and the water (solvent).
With constant stirring the solute molecules get evenly dispersed through the solvent in a uniform mixture of the two. In a dilute solution as described above, solute does not settle down but remains evenly dispersed. The solution may be dilute or concentrated depending upon the quantity of the solute added in the solvent.

At a given temperature and pressure, only a specific quantity of solute can be dissolved in a given solvent. The solution in such situations is said to be saturated. In our example, it may be stated that both common salt and sucrose form solutions but these are of different kinds. For instance, sucrose is a non-ionic substance whereas common salt is an ionic substance.

This means that while the former remains intact in water the latter ionizes in water. In other words, common salt (NaCl) breaks into Na+ and Cl^-ions in water and these are evenly distributed. As a result a true solution is formed.

Depending open the solvent whether it is liquid, solid or gas, following categories of solution may be formed.

(a)	solid—liquid solid—solid solid—gas	(b)	liquid—liquid liquid—solid liquid—gas	(c)	gas—liquid gas—solid gas—gas

Water is the most common solvent in the plant and animal cell and its environment. It is universally available and has specific characteristic features. It has highest boiling point

and is highly polarized and behaves like dipoles. The heat of vaporization of H_2O is unusually high. Also, water has high surface tension and this accounts for its capillary action.

Three types of solutions occur in the cells:

(i) **Solution of a gas in a liquid**, carbon dioxide, nitrogen and oxygen are commonly found as gases in solution.

(ii) **Solution of liquids in liquids** fall into *two classes*; those in which liquids are freely miscible in all proportions e.g., ethyl alcohol in water and secondly those in which two liquids are sparingly soluble e.g. ether, chloroform in water. Ether and water when shaken thoroughly in a flask and then allowed to stand form distinct layers they are immiscible. The upper layer will be a dilute solution of water in ether and the bottom layer will be a dilute solution of ether in water. The liquid-in-liquid solution is where components are freely miscible; the component in greatest amount is called the solvent.

(iii) **Solution of a solid in a liquid** is the common type of solution, e.g., solution of sucrose and common salt (NaCl) in water. Sucrose molecule is non-ionic and, therefore, remains as such in water. Sodium chloride (NaCl), on the other hand, is an ionic compound and undergoes ionization in water. Thus, the sodium chloride molecule breaks down to form sodium and chloride ions. These molecules or ions are evenly distributed throughout the water, forming a stable homogenous mixture called a true solution.

Suspension: In a suspension, the particles are greater than 1 μm in size and the particles do not separate into molecules but are aggregates of molecules which can be seen with naked eye. It is an unstable system. When allowed to stand, particles gradually settle down at the bottom of the container separated by a liquid layer. The most common example of suspension is sand in water.

Colloidal System: The sizes of the dispersed particles as well as the properties of the system are midway between the suspension and the true solutions. The size of particles ranges from 0.001 μm to 0.1 μm in diameter and they remain dispersed throughout water in a stable manner, forming a two-phase system.

The liquid phase is called dispersion phase while the solid phase is called the dispersed phase. The colloidal particles are in the form of clusters of molecules. They can be seen under the electron microscope.

<u>Expression of Concentration of a Solution</u>: Concentration of a solution is the relative proportion of the solute in relation to solvent. **It is expressed by any one of the following methods:**

1. Percentage Solution (%) (w/v):
The volume or weight of the solute is calculated as percent of the solution.
This is shown below:
10% sodium chloride = 10 g NaCl dissolved in water till a final volume of 100 ml

2. Molarity (M): Molarity (M) is defined as the number of moles of solute per liter of solution. molarity = moles of solute/liters of solution

3. Molality (m): Molality (m) is defined as the number of moles of solute per kilogram of solvent. molality = moles of solute/kilograms of solvent

Although their spellings are similar, molarity and molality cannot be interchanged. Molarity is a measurement of the moles in the total volume of the solution, whereas molality is a measurement of the moles in relationship to the mass of the solvent.

When water is the solvent and the concentration of the solution is low, these differences can be negligible (d = 1.00 g/mL). However, when the density of the solvent is significantly different than 1 or the concentration of the solution is high, these changes become much more evident.

Example:

Compare the molar and molal volumes of 1 mol of a solute dissolved in CCl_4 (d = 1.59/mL).

For a 1 Molar solution, 1 mol of solute is dissolved in CCl_4 until the final volume of solution is 1 L.

For a 1 molal solution, 1 mol of solute is dissolved in 1 kg of CCl_4.
1 kg of CCl4 × (1,000 g/1 kg) × (mL/1.59 g) = 629 mL CCl_4

4. Normality (N): A normal solution of a substance is obtained by dissolving a gram equivalent weight of that substance in a litre of solution. If we dissolve 2 g equivalent weight in a litre of solution we get a 2N (two normal) solution.
The concentration of acid and alkali solutions is more easily expressed in terms of normality than molarity.

The gram equivalent weight of an acid or base is the quantity that will release or neutralize mole of an hydrogen ions. 1M solution of HCl is also a 1N solution of the acid.

However, 1M solution of H_2SO_4 would be 2N since it is capable of releasing 2 moles of hydrogen ions.

5. Parts per Million (ppm):

A gram of solute per million grams of solution or gram of solute per million ml of solution

$$\text{ppm} = \frac{\text{Mass of component}}{\text{Total mass of solution}} \times 10^6$$

or

$$\text{ppm} = \frac{\text{ml of solute}}{\text{ml of solution}} \times 10^6$$

NaCl (1 ppm) in water

1 ppm = 1 mg NaCl/L of solution

= 1 mg NaCl/1000 ml of solution.

- **<u>Ideal and non-ideal Solution:</u>**

The solutions which obey Raoult's law at all compositions of solute in solvent at all temperature are called ideal solution.

Ideal solutions can be obtained by mixing two components with identical molecular size, structure and they should have almost same inter molecular attraction e.g., two liquids A and B form and ideal solution when A – A and B – B molecular attractions will be same and hence A-B molecular attraction will be almost same as A-A and B-B molecular attraction.

Examples of Ideal solutions: Ethyl chloride and ethyl bromide; n-hexane and n-heptane; CCl_4 and $SiCl_4$.The solution which deviate from ideal behaviour are called non ideal solution or real solutions and they do not obey Raoult's law over entire range of composition. It has been found that on increasing dilution, a non ideal solution tend to be ideal.Such a solution show positive deviation from Raoult's Law and the observed boiling point of such solutions is found to be less than the calculated value.e.g. cyclohexane and ethanol.In ethanol the molecules are held together due to hydrogen bonding, when cyclohexane is added to ethanol the molecules of cyclohexane tend to occupy the space between ethanol molecules due to which some hydrogen bonds break due to which inter molecular attraction between cyclohexane and ethanol is reduced and hence show higher vapour pressure.Solutions of above type show negative deviation from Raoult's law and their observed boiling point is found to be higher than the calculated value. For example, when acetone and chloroform are mixed together a

hydrogen bond is formed between them which increases inter molecular attraction between them and hence decreases the vapour pressure.

Probable questions:

1. Define molarity, normality and molality.
2. What is solution? How many types of solutions are there?
3. What is suspension?
4. What is idea or non ideal solution? Give example.

Suggested readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

UNIT II

Chemical & physical effects of some primary fixatives: Formalin, alcohol, picric acid, acetic acid

Objective:

In this unit we will discuss about Chemical & physical effects of some primary fixatives: Formalin, alcohol, picric acid, acetic acid

Fixatives:

A large number of chemicals such as ethyl alcohol, formalin, acetic acid, chloroform, mercuric chloride, chromic acid, picric acid, osmic acid, etc. are used singly or in combinations as fixatives for anatomical studies. Amongst these, formalin and formalin-aceto-alcohol (FAA) are most commonly used in Anatomy.

Fixation is the first or the foundation step of the cell/histotechniques done to preserve the tissues in as close a life like state as possible by preventing their autolysis and putrefaction. A number of fixatives exist, either having being in use for decades, or in the case of formaldehyde over a century. Every fixative has different properties, each indicated for a special purpose based on the type of cell component to be studied, the method of sectioning and staining employed and the type of microscopy involved. Thus, a pathologist must have a fair idea of the properties of these commonly available fixatives, so that a correct choice can be made depending upon the desiredresults. This review aims to give a brief overview of the commonly available fixatives with their merits and their demerits.

The goal of fixation is to preserve structure as faithfully as possible compared to the living state. The three most important parameters to remember about fixation are:

(1) Keep the time between killing and fixation to a minimum.

(2) Keep the size of the tissue as small as possible without losing information or destroying the tissue. If a large specimen must be fixed, keep one dimension less than 1 mm, or else nick areas of the specimen that can be discarded so that the fixative can penetrate.

(3) Keep gross tissue deformation to a minimum by using sharp implements and keeping manipulation of the specimen to the minimum necessary.

Properties of Fixatives and Factors Affecting Fixation

1. Coagulation and precipitation of proteins in tissues.

2. Penetration rate differs with different fixatives depending on the molecular weight of the fixative

3. pH of fixatives – Satisfactory fixation occurs between pH 6 and 8. Outside this range, alteration in structure of cell may take place.

4. Temperature – Room temperature is alright for fixation. At high temperature there may be distortion of tissues.

5. Volume changes – Cell volume changes because of the membrane permeability and inhibition of respiration.

6. An ideal fixative should be cheap, nontoxic and non-inflammable. The tissues may be kept in the fixative for a long time.

Aims of Fixation

(a) To preserve the tissues as close to their living state as possible

(b) To prevent autolysis and bacterial attack

(c) To prevent tissues from changing their shape and size during processing

(d) To harden the tissues

(e) To allow clear staining of sections subsequently

(f) To improve the optical differentiation of cells & tissues

- **Formalin Fixative :**

Although various fixatives are available and the information about each fixative is extensive, our focus will be on formalin fixation. The most common and widely accepted fixative is 10% neutral buffered formalin (NBF). This aqueous solution is considered a "universal fixative" since it can be used for a wide variety of stains and techniques.

The properties of formalin are as follows:

- ✓ Penetrates tissue quickly, but fixes slowly because cross-linking to tissue proteins takes a long time.
- ✓ Causes less tissue shrinkage than other fixatives.
- ✓ Hardens tissue, but less than alcohols and acetone.
- ✓ Relatively inexpensive and stable.

- ✓ Lipids are preserved, but not made insoluble.
- ✓ May result in formalin pigment in bloody tissue, but this is prevented by neutralizing the formalin.

Although biopsy specimens may only require several hours to fix properly, larger surgical samples require eight or more hours of fixation prior to the subsequent processing steps. Most small specimens are fixed at room temperature prior to processing, but larger samples will finish fixing in the first step on the tissue processor. Since formalin and paraffin are not miscible (capable of mixing), formalin-fixed tissue must be taken through several intermediate steps before it is placed in paraffin.

- **95% Alcohol**

PURPOSE: Used as a fixative for frozen sections.

- **Bouin's solution (Yellow)**

Composition: Picric acid, formaldehyde, and acetic acid.

Formulation

I. Picric acid saturated aqueous solution. (2.1%): 750 ml
II. 40% formaldehyde: 250 ml
III. Acetic acid glacial: 50 ml
IV. Fixation time: 4 – 18 hours

- **Recommended Applications**

Gives very good results with tissue that is subsequently trichrome stained. Preserves glycogen well but usually lyses erythrocytes. Sometimes recommended for gastrointestinal tract biopsies, animal embryos, and endocrine gland tissue. Stains tissue bright yellow due to picric acid. Excess picric should be washed from tissues prior to staining with 70% ethanol. Because of its acidic nature, it will slowly remove small calcium deposits and iron deposits.

- **Advantages:**

Fixation in Bouin's will result in sharp Haematoxylin and Eosin staining and is preferred by some pathologists. Bouin's fixation can facilitate finding small lymph nodes. The nodes will remain white and the fat is stained yellow. Prolonged fixation can be used to decalcify tissue.

- **Disadvantages:**

Tissues will become quite brittle and should not be fixed for over 18 hours. Tissues can be transferred to ethanol to avoid this. Large specimens should not be fixed in Bouin's as it will color the entire specimen yellow and it will be difficult to see details grossly. Red cells will be lysed and iron and small calcium deposits dissolved. Immunoperoxidase studies performed on tissues fixed in Bouin's may be less sensitive. Picric acid can cause degradation of DNA and RNA and may interfere with the use of tissues for special studies requiring intact DNA, such as PCR (polymerase chain reaction).

- **Acetic acid**

Acetic acid (CH_3COOH) is coagulant in action with nucleic acids but generally does not fix proteins. It is incorporated in compound fixatives to help prevent the loss of nucleic acids and, because it swells collagen, to counter the shrinkage caused by other ingredients such as ethanol. Acetic acid penetrates very rapidly but fixatives that contain it will lyse red blood cells.

Probable questions:

1. What is fixative? Give example.
2. What is fixation?
3. Describe the purpose of fixation.
4. What is the composition of Formalin Fixative?
5. Write down the properties of formalin.
6. Write down the names and mechanism of action of 2 commonly used fixatives.
7. State the advantages and disadvantages of Bouin's fixative.

Suggested readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

UNIT III

Source and chemical composition of some dyes: Basic fuchsin, carmine, hematin, hematoxylene, eosin.

Objective:

In this unit you will learn aboutSourceand chemical composition of some dyes: Basic fuchsin, carmine, hematin, eosin

Stains:

A stain is any colouring organic compound that, when combined with another substance, imparts a colour to that substance. The terms 'dyes' and 'stains' are often used interchangeably by biologists, but they are not the same. The term 'dye' is used to refer to a colouring agent that is used for general purposes, whereas the term 'stain' is used to refer to that dye which is used for biological purposes.

Most of the stains used, particularly for bacteria, are aniline dyes, so called because their derivation from aniline ($C_6H_5NH_2$). The most commonly used aniline dyes are crystal violet, methylene blue, basic fuchsin, safranin, eosin, etc.

Purpose of Staining:

Staining is done for any or all of the following three purposes:

(a) To see organism better:

Staining enables to see the organism better in contrast with background.

(b) To differentiate one organism from another:

Some microorganisms take colour under the given staining conditions, some do not. Such differences are particularly evident in staining procedures which are therefore called "differential stains", the most common differential stains being the Gram- stain and the acid-fast stain,

(c) To determine particular structures:

There are special stains which react only with certain structures of the organism, e.g., spores, cell wall, nuclei, or others. This is why an organism stained with a cell wall-stain shows only the presence or absence of its cell wall.

Structural Components (Nature) of Stains:

Stains (dyes) usually have complex molecular structure and are chiefly benzene derivatives. A stain consists of three constituents: the organic compound containing a benzene ring, the chromophore, and the auxochrome. Thus a stain may be defined chemically as an organic compound containing both chromophore and auxochrome groups linked to its benzene ring.

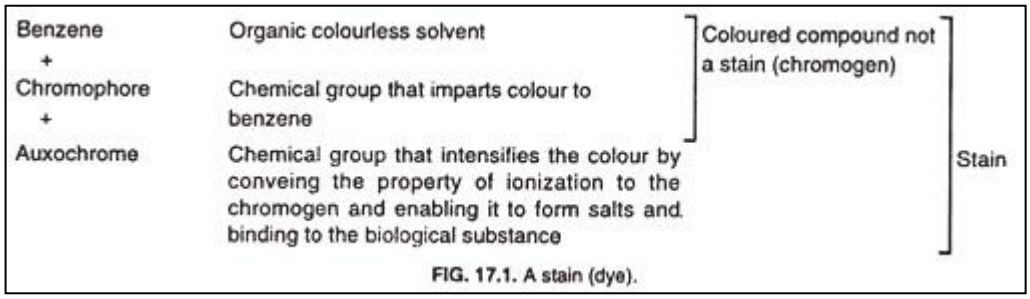


FIG. 17.1. A stain (dye).

Mordant and Its Function:

Mordant is a substance that forms an insoluble compound with a stain and helps to fix the colour to the cell components. Some stains never stain the cells or its components unless treated with a mordant. The mordant becomes attached to the cell or its components and then combines with the stain to form an insoluble colour complex. This complex is called a lake.

Commonly used mordants are the oxides of aluminium, iron, and chromium. Alizarin is an example of a stain that imparts colour only in collaboration with a mordant. It gives different colours when used with different mordents. It gives red colour with aluminium and tin salts, brownish red colour with a chromium mordant, and black-violets with an iron mordant.

Basic fuchsine:

Basic fuchsine is a mixture of rosaniline, pararosaniline, new fuchsine and Magenta II. Formulations usable for making of Schiff reagent must have high content of pararosanilin. The actual composition of basic fuchsine tends to somewhat vary by vendor and batch, making the batches differently suitable for different purposes.

In solution with phenol (also called carbolic acid) as an accentuator it is called carbol fuchsin and is used for the Ziehl–Neelsen and other similar acid-fast staining of the *Mycobacteria* which cause tuberculosis, leprosy etc. Basic fuchsine is widely used in biology to stain the nucleus, and is also a component of Lactofuchsin, used for Lactofuchsin mounting.

Acetocarmine:

Dry carmine powder … 0-5-1 -0 g

Distilled water … 55 ml

Glacial acetic acid … 45 ml

Preparation: Mix 45 ml glacial acetic acid and 55 ml distilled water and heat to boiling in a conical flask with cotton plugging. Add 0.5 to 1.0 g carmine powder. Shake well, cool and filter and store as stock solution.

Acetocarmine is a non-specific nuclear stain which simply binds the chromosomes and gives colour to them. However, specific nuclear stain (e.g. feulgen) reacts with chromosomes to give them the colour. Acetocarmine is a dye obtained from insects.

Delafield's Haematoxylin:

Reagents:

Saturated aqueous ammonia solution … 400 ml

Haematoxylin … 4 g

90% ethyl alcohol … 25 ml

90% methyl alcohol … 100 ml

Glycerine … 10 ml

Preparation: Dissolve 4 g haematoxylin in 25 ml 90% ethyl alcohol. Mix the solution with 400 ml ammonia solution. Leave the mixture exposed to air and light for 4 days. Add 100 ml 90% methyl alcohol and 10 ml glycerine. Filter and leave to ripen for about 2 months in a bottle plugged with cotton wool.

This haematoxylin is used for staining protozoan parasites. The protozoans are mordented in 3% iron alum first, then stained with haematoxylin.

Hematoxylin and eosin (H&E) stains have been used for at least a century and are still essential for recognizing various tissue types and the morphologic changes that form the basis of contemporary cancer diagnosis. The stain has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features. Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Well-fixed cells show considerable intranuclear detail. Nuclei show varying cell-type- and cancer-type-specific patterns of condensation of heterochromatin (hematoxylin staining) that are diagnostically very important. Nucleoli stain with eosin. If abundant polyribosomes are present, the cytoplasm will have a distinct blue cast. The Golgi zone can be tentatively identified by the absence of staining in a region next to the nucleus. Thus, the stain discloses abundant structural information, with specific

functional implications. A limitation of hematoxylin staining is that it is incompatible with immunofluorescence. It is useful, however, to stain one serial paraffin section from a tissue in which immunofluorescence will be performed. Hematoxylin, generally without eosin, is useful as a counterstain for many immunohistochemical or hybridization procedures that use colorimetric substrates (such as alkaline phosphatase or peroxidase). This protocol describes H&E staining of tissue and cell sections.

Eosin (Aqueous):

Reagents:

Eosin powder ….. 1 g

Distilled water ….. 100 ml

Preparation: Dissolve the eosin powder in 100 ml distilled water.

Eosin is a class of fluorescent red dye. It is an artificial derivative of fluorescein consisting of two closely related compounds, eosin Y and eosin B. Eosin Y is far more commonly used. It is a tetrabromo derivate of fluorescein and has a slightly yellowish cast (so is also known as Eosin Yellowish). Eosin Y can be further divided into water-soluble and ethanol-soluble eosin Y.

Probable questions:

1. What are the basic components of a stain?
2. What is mordant?
3. Mention the composition of haematoxylin and eosine.

Suggested readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

UNIT IV

Chemical composition and properties of fluorescence dye, principle and application: DAPI, Propidium Iodide, Acridine orange, Rhodamine, DCFDA, Hoechst

Objective:

In this unit we will discuss about Chemical composition and properties of fluorescence dye, principle and application: DAPI, Propidium Iodide, Acridine orange, Rhodamine, DCFDA, Hoechst.

Fluorescence Dye

Introduction

Fluorescent dyes (also known as fluorophores/reactive dyes) may simply be described as molecules (non-protein in nature) that, in microscopy, achieve their function by absorbing light at a given wavelength and re-emitting it at a longer wavelength. This produces fluorescence of different colors that can be visualized and analyzed.

As a result, they are often used in fluorescent labeling of various biomolecules (antibodies, peptides, various proteins, etc) for such processes as monitoring the delivery of drugs to targeted tissues, imaging, among others.

While proteins (e.g. green fluorescent protein) contain fluorescent amino acids (tryptophan, tyrosine, and phenylalanine), they offer photostability and brightness compared to fluorescent dyes. Unlike fluorescent dyes, they also require maturation time thus making them less ideal.

Properties of Fluorescent Dyes are as follows:

- Not alter the general shape and function of the target molecules/cells
- Be localized at the target location on the cell/molecule
- Maintain high specificity even in the presence of other molecules
- Operate at visible wavelengths
- Fluorescent dyes are divided into several groups that include: Organic dyes
- Biological fluorophores
- cover the whole UV/VIS spectrum with all colors available

- fit the common commercial light sources and filter systems
- have superiour properties with primary focus on
    - signal intensity
    - high solubility in water
    - photostability (minimal photobleaching)
    - low molecular weight resulting in minimal steric hindrance

Chemical composition of Fluorescent Dyes

It is a xanthene dye, a gamma-lactone, a polyphenol, an oxaspiro compound, a member of 2-benzofurans and an organic heteropentacyclic compound. It is functionally related to a fluorescein (acid form).

- **Organic Fluorescent Dyes**

Essentially, organic dyes are characterized by emissions originating from optical transition delocalized over the whole chromophore or from intermolecular charge transfer transitions (intramolecular charge transfer from the excited electronic state).

Here dyes that exhibit an emission originating from optical transition delocalized over the whole chromophore are known as resonant dyes (mesomeric dyes) while the latter are referred to as CT dyes (Charge transfer dyes).

Cyanines, rhodamines, and fluoresceins which are some of the most common resonant dyes are characterized by narrow absorption and emission bands (which are slightly structured) that tend to mirror each other as well as small, slightly solvent polarity-insensitive Stokes shift.

Fluorescein

Like cyanines and rhodamine dyes, fluorescein is also an organic dye. With an absorption maximum at 494nm and emission maximum of 521nm (typically absorbing light in the blue range and emitting it in the green range), fluorescein is a highly fluorescent substance. It can be detected even when in very small quantities and is used in microscopy when it's conjugated to antibodies.

Derivatives of fluorescein include fluorescein isothiocyanate, Oregon Green, and carboxynaphthofluorescein among a few others. Like a number of other fluorescent dyes, fluorescein is inexpensive and easy to use making it one of the most popular dyes in biological research.

Unlike most of the other dyes, fluorescein is nontoxic in aqueous solutions. As a result, it is one of the very few dyes used as a groundwater tracer.

Principle and application of DAPI, Propidium Iodide, Acridine orange, Rhodamine, DCFDA, Hoechst

1. 4',6-diamidino-2-phenylindole (DAPI)

A simple-to-use fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI), visualizes nuclear DNA in both living and fixed cells. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology.

Principle: It is believed that DAPI associates with the minor groove of double-stranded DNA, with a preference for the adenine-thymine clusters. Cells must be permeabilized and/or fixed for DAPI to enter the cell and to bind DNA. Fluorescence increases approximately 20-fold when DAPI is bound to double-stranded DNA.The procedure is quick, easy to use, inexpensive, and can be used as a preliminary or quantitative method to detect or quantify phytoplasma-like bodies in infected plants.

Application: DAPI is generally used to stain fixed cells since the dye is cell impermeant, although the stain will enter live cells when used at higher concentrations. For live-cell staining, Hoechst 33342 dye is a popular cell-permeant nuclear counterstain.

2. Propidium iodide (PI):

Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain. Since propidium iodide is not permeant to live cells, it is also commonly used to detect dead cells in a population. PI binds to DNA by intercalating between the bases with little or no sequence preference.

Principle: Propidium iodide (PI) is a cell-impermeant DNA binding dye that can be used to stain cells and nucleic acids. PI binds to DNA by intercalating between the bases with a stoichiometry of one dye per 4-5 base pairs of DNA. Little or no sequence preference is observed. Free PI has excitation/emission maximums of 493/636 nm, respectively. Once bound to DNA, the excitation/emission maximum is shifted to 535/617 nm, and its quantum yield is enhanced 20-30 fold, exhibiting bright orange-red fluorescence.

As a membrane impermeant dye, PI is generally excluded from viable cells. However, it can penetrate the dead cells, which have compromised membrane integrity, and stain the intracellular double-stranded DNA molecules. Therefore, PI is widely used to evaluate cell viability, measure DNA content in cell cycle analysis, as well as visualize the nucleus and other DNA-containing organelles.

3. Acridine orange

Acridine orange is a fluorescent dye which easily traverses the cell membrane. Because of its weak basic property, it accumulates in lysosomes, which have a low pH inside, due to an ATP-dependent proton pump, present in their membrane. AO has metachromatic properties and upon excitation with blue light, (~488 nm) emits green fluorescence when in monomer form and orange fluorescence when in dimer form. AO produces orange fluorescence when it binds to RNA and green fluorescence when it binds to DNA.

Principle: Acridine orange is a fluorochromatic dye which binds to nucleic acids of bacteria and other cells that causes deoxyribonucleic acid (DNA) to fluoresce green and ribonucleic acid (RNA) or single stranded DNA to fluoresce orange-red under UV light. It was first described by **Strugge and Hilbrich** in 1942. When buffered at pH 3.5 to 4.0, acridine orange differentially stains microorganisms from cellular materials. It has been recommended for the rapid identification of *Trichomonas vaginalis*, yeast cells, and clue cells in vaginal smears. Intracellular gonococci, meningococci, and other bacteria particularly in blood cultures are also detected. In clinical samples, Bacteria and fungi stain bright orange whereas, Human epithelial and inflammatory cells and background debris stain pale green to yellow. Nuclei of activated leukocytes stain yellow, orange, or red due to increased RNA production resulting from activation. Erythrocytes either do not stain or appear pale green. The acridine-orange stain is an optional stain that can be helpful in detecting organisms not visualized by Gram stain, often due to a large amount of host cellular debris.

Application: Acridine orange is useful in the rapid screening of ordinarily sterile specimens. When acridine orange is used with flow cytometry, the differential stain is used to measure DNA denaturation and the cellular content of DNA versus RNA in individual cells, or detect DNA damage in infertile sperm cells.

4. Rhodamine

Rhodamine B /ˈroʊdəmiːn/ is a chemical compound and a dye. It is often used as a tracer dye within water to determine the rate and direction of flow and transport. Rhodamine dyes fluoresce and can thus be detected easily and inexpensively with fluorometers.

Along with eosin and fluorescein, rhodamine belongs to the xanthenes family. As compared to a number of other dyes in the market, rhodamines exhibit excellent photostability as well as a number of photophysical properties making them ideal for use as laser dyes, fluorescent probes, and pigments.

Application: Rhodamine dyes are used extensively in biotechnology applications such as fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy and ELISA. Rhodamine 123 is used in biochemistry to inhibit mitochondrion function.

Rhodamine auramine stain is used for the detection of mycobacteria directly from clinical specimens. The dye binds with the mycolic acids and fluoresces under ultraviolet light. Acid fast organisms (mycobacteria) will appear yellow or orange under ultraviolet light.

5. Hoechst dyes

Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. Hoechst 33342 binds preferentially to adenine-thymine (A-T) regions of DNA. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye:base pair ratios.

Principle: Hoechst dyes are popular blue fluorescent, nuclear-specific dyes used to stain live or fixed cells. They bind preferentially to adenine-thymine (A-T) regions of DNA and exhibit distinct fluorescence emission spectra dependent on dye:base pair ratios. Hoechst dyes are excited by UV light (~360 nm) or mercury-arc lamps, or UV lasers and emit a broad spectrum of blue light with a maximum in the 460 nm region. The dyes have minimal fluorescence in solution, but their fluorescence increases ~30-fold upon DNA binding, which ensures a good signal-to-noise ratio. Therefore, they can be used to stain cells without a wash step. The staining is very stable and non-toxic.

Hoechst 33342 and Hoechst 33258 are structurally similar dyes that perform comparably as nuclear counterstains. Hoechst 33528 is slightly more water soluble than Hoechst 33342, but both dyes are highly cell membrane-permeant and widely used in cell cycle studies and as nuclear counterstains for live or fixed cells. They are typically used for staining at 1 ug/mL.

Application: Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells and tissues. This stain is commonly used in combination with 5-bromo-2'-deoxyuridine (BrdU) labeling to distinguish the compact chromatin of apoptotic nuclei, to identify replicating cells and to sort cells based on their DNA content. Hoechst 33342 and propidium iodide are frequently used together for simultaneous flow cytometric and fluorescence imaging analysis of the stages of apoptosis and cell-cycle distribution.

6. DCFDA

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (also known as dichlorofluores *cin* diacetate) is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells, for example to detect the generation of reactive oxygen intermediates in neutrophils and macrophages. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is

converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Versions of this indicator that have better cellular retention are available

Application: DCFDA is used both in mitochondrial preparations and live cell imaging. MitoTracker Red (CM-H2XRos) can accumulate within mitochondria in response to the positive charge acquired by oxidation by ROS and is retained in the organelle by covalently binding to mitochondrial proteins.

Probable questions:

1. What is Fluorescent Dye?
2. Mention the properties of Fluorescent Dyes.
3. What is the chemical nature of Fluorescent Dye.
4. What is an organic fluorescent dye?
5. Write short notes on fluorescein.
6. What is the use of DAPI in fluorescence microscopy?
7. What is the application of DAPI staining?
8. Describe the structure of Acridine orange.
9. What is the principle of Acridine orange activity?
10. Write down the principle and application of Rhodamine dye.
11. Write down the principle and application of Hoechst dyes
12. Write down the principle and application of DCFDA.

Suggested readings:

1. Advanced Practical Zoology. Sinha JK, Chatterjee AK & Chattopadhyay P. NCBA.
2. Junqueira's Basic Histology text and atlas. Anthony M. McGraw Hill Lange.
3. Histochemistry in focus. Shyamsundari K &Hanumantha Rao K. MJP.
4. Principles and techniques of biochemistry and molecular biology. Wilson K & Walker J. Cambridge University Press.

UNIT V

Study of subcellular organelle under light microscopy and electron microscopy

Objective: In this unit we will discuss about Study of subcellular organelle under light microscopy and electron microscopy.

Introduction

An organelle is a subcellular structure that has one or more specific jobs to perform in the cell, much like an organ does in the body. Among the more important cell organelles are the nuclei, which store genetic information; mitochondria, which produce chemical energy; and ribosomes, which assemble proteins.

Subcellular organelles that are connected by pathways of membrane traffic include (1) the ER, (2) the nucleus, (3) the Golgi apparatus, (4) various endosomes, and (5) lysosomes. The ER has the largest surface area of any subcellular organelle and is the site where most proteins and lipids are first inserted into a membrane.

1. Nucleus

The largest organelle in the cell is nucleus which is enveloped by bound double layered nuclear membrane preserving the genetic material called chromatin. Nucleus occupies 1%-2% and 10% in yeast and animal cells respectively. The genetic material forms a mass called chromatin that is concentrated in one part of the nucleus. The outer and inner membranes are separated by lumen. The outer membrane of the nuclear envelope is continuous with the endoplasmice reticulum (ER) membrane, and the lumen of the nuclear envelope is continuous with the lumen of the ER. The inner nuclear membrane is usually supported by a network of filaments called the nuclear lamina, located in the nucleus and anchored to the inner membrane. The nucleus contains subcompartments with specialized functions and the major subcompartment in the nucleus is the nucleolus.

The pores of nuclear membranes are large enough to be completely permeable to smaller molecules, so there is no difference in the aqueous environment of the nucleus and the cytoplasm. The nucleus is considered to be the core of the cell which regulates all metabolic events.

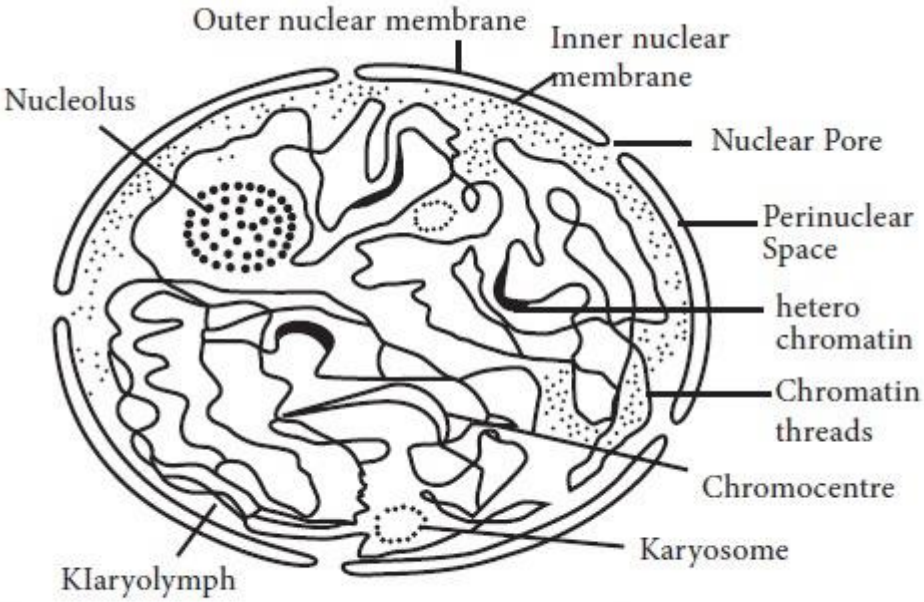


Figure 1.6 A diagrammatic representation of the ultrastructure of nucleus

Nuclear envelope: The nucleus is separated from the cytoplasm by a double membrane, the nuclear envelope and the two membranes separated from each other by a perinuclear space of varying width. There are little holes in the nuclear envelope called nuclear pores which help the substances to move into or out of the nucleus. DNA occupies most of the space inside a nucleus. DNA is the genetic material and provides the instructions essential for building proteins. Proteins are responsible for helping with most activities in a cell. Inside the nucleus is a round body called nucleolus, which is present in a eukaryotic cell. The nucleolus is devoid of an encircling membrane. The nucleolus produces the ribosomal subunits from proteins and ribosomal RNA, also known as rRNA. It then sends the subunits out to the rest of the cell where they combine into complete ribosomes. Ribosomes make proteins; therefore, the nucleolus plays a vital role in making proteins in the cell.

Electron micrograph of Nucleus

This picture shows an electron micrograph of a nucleus. The short white arrows are pointing to nuclear pores. Note the appearance of eu- and heterochromatin, and the nucleolus. Heterochromatin stains more densely than euchromatin, but they are both forms of chromatin. Chromatin is the name for the diffuse granular mass of DNA found in interphase cells.

Heterochromatin is less abundant, relative to **euchromatin**, in the large nuclei of active cells than in the small nuclei of resting cells, such as small lymphocytes.

Euchromatin is "active" chromatin, containing DNA sequences that are being transcribed into RNA.

The **nucleolus** is the site in the nucleus where ribosomal RNA is transcribed. It is then linked to the subunits of the ribosome, and transported out of the nucleus through nuclear pores. The ribosomes assemble, and translation of RNA and protein synthesis occurs in the cytoplasm. Protein synthesis occurs on free ribosome or on ribosomes attached to the endoplasmic reticulum (rough endoplasmic reticulum), in which case a pore is formed so that newly synthesized proteins move into the cisterna of the rough endoplasmic reticulum. The proteins synthesised on ribosomes attached to the ER, are then transported to the Golgi, and packaged for secretion.

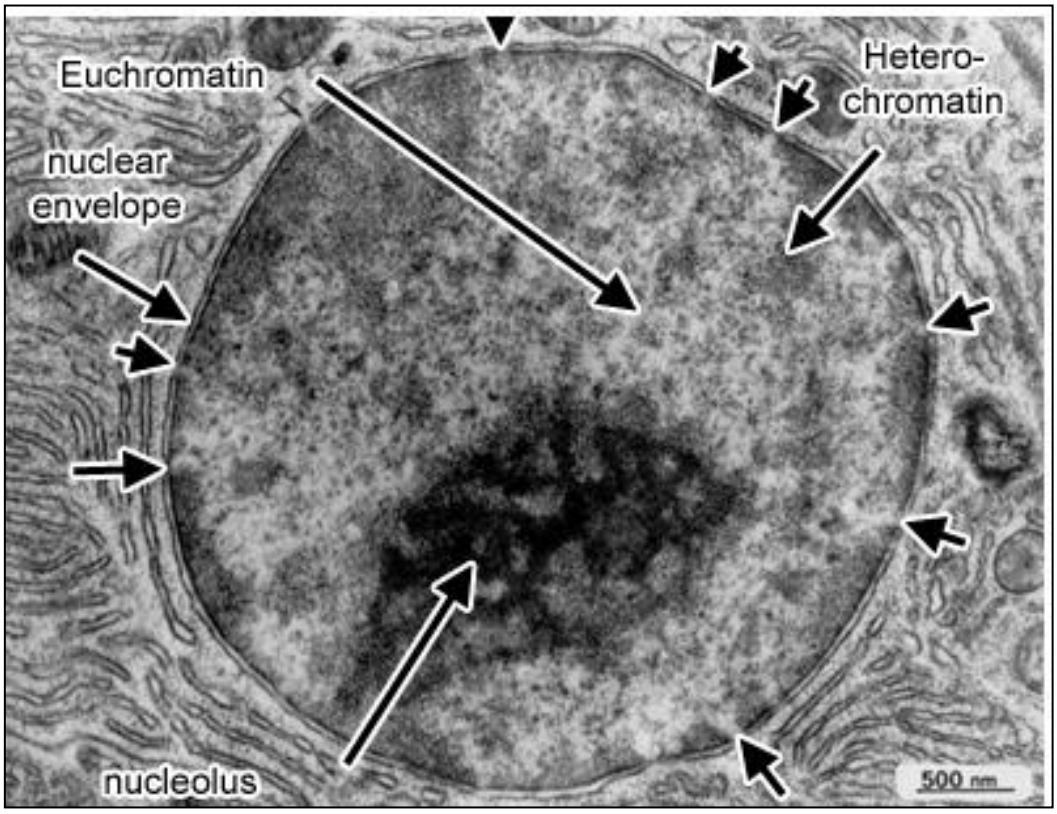


Fig: An electron micrograph of a nucleus

2. Mitochondria- the power houses of the cell

A cell has a compartment for energy production. It obtains energy from the food supplied by its environment. This energy then has to be converted into some form that can be distributed throughout the cell. The common solution is to store energy in the form of a common molecule that can be used whenever and wherever it is needed in the cell. The term 'mitochondrion' is derived from the Greek word 'mitos' which means 'thread' and 'chondrion' which means 'granule'. Mitochondria are a membrane bound cellular structure and is found in most of the eukaryotic aerobic cells. Mitochondria may assume different shapes ranging from granular to filamentous depending upon the functional state of the cell. They are spherical in yeast cells, elliptical in kidney cells,

elongated in liver cells and filamentous in fibroblasts. The size of the mitochondria ranges from 0.5 to 1.0 μm in diameter.

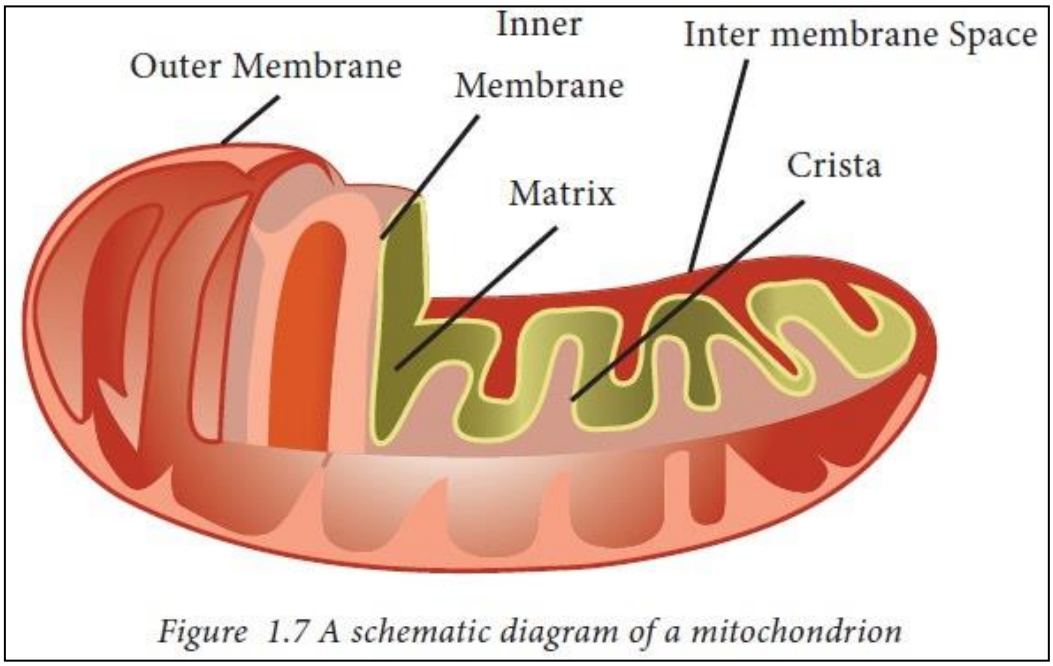


Figure 1.7 A schematic diagram of a mitochondrion

The mitochondria consist of a smooth outer membrane, which has a large number of special proteins known as the porins, separated by a space from an inner membrane. The inner membrane is thrown into folds or invagination called cristae which extend into matrix, the mitochondrial lumen. Both the membranes are separated by a clear inter membrane space. The cristae are irregularly shaped like villous and finger like projections. The membranes are made up of phospholipids and proteins.

Functions of Mitochondria

a. The mitochondria can help the living cell to convert energy supplied by the environment into ATP, the common molecule, required for chemical reactions. ATP can be generated in two pathways: in the cytosol, and in mitochondrion. First pathway exists in the cytosol of an eukaryotic cell (or within a bacterial cell) where glycolysis degrades glucose to lactate and releases two molecules of ATP.

b. Second pathway is the main source of energy production as ATP (called oxidative phosphorylation and involves the electron transport chain). Pyruvate generated from glycolysis enters the matrix (lumen) of the mitochondrion, where it is degraded and combined with coenzyme A to form acetyl CoA. The acetyl part of the acetyl CoA is then degraded to carbon dioxide by the citric acid cycle, releasing hydrogen atoms. The hydrogen atoms are used to reduce the carrier NAD^+ to NADH, and then oxidation of NADH releases a proton and an electron.

c. Mitochondria help the cells to maintain proper concentration of calcium ions within the compartments of the cell.

d. Mitochondria also help in erythropoiesis and biosynthesis of hormones like testosterone and estrogen.

e. The mitochondria of liver cells have enzymes that detoxify ammonia.

f. The mitochondria also play an important role in the process of apoptosis or programmed cell death. Abnormal death of cells due to the dysfunction of mitochondria can affect the function of an organ.

g. The mitochondria are involved in other cellular activities like signalling, cellular differentiation and cell senescence. They also regulate the control of cell cycle and cell growth.

h. Unlike the outer membrane, the inner membrane is strictly permeable, it is permeable only to oxygen, ATP and it also helps in regulating transfer of metabolites across the membrane.

i. The matrix of the mitochondria is a complex mixture of proteins and enzymes. These enzymes are important for the synthesis of ATP molecules, mitochondrial ribosomes, tRNAs and mitochondrial DNA.

j. Mitochondria also affect human health. Mitochondrial disorders and cardiac dysfunction also play an important role in the aging process.

3. Endoplasmic reticulum (ER):

Eukaryotic cells contain several interrelated membrane-bound compartments, collectively termed as 'endomembrane system' or ER.It is a continuous membrane, which is present in both plant cells, animal cells and absent in prokaryotic cells.There is a series of convoluted membrane sheets which are contiguous with the outer membrane of the nuclear envelope. This series of membrane delimited compartments in a typical eukaryotic cell are related and interact with one another by fission and fusion of their membranes. The space, which is present in the endoplasmic reticulum, is called as the lumen.

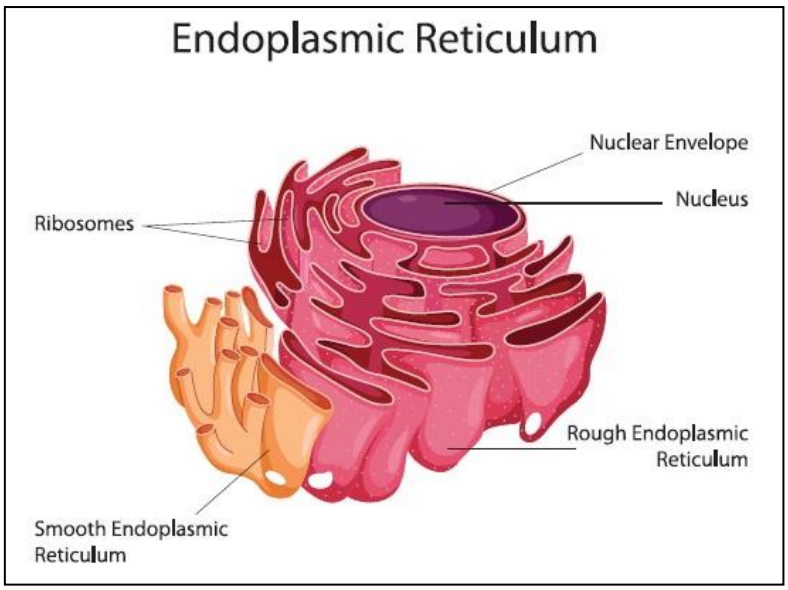
There are three morphological patterns in ER.

a. Granular or Rough endoplasmic reticulum

b. Smooth endoplasmic Reticulum

c. Lamellar and Vesicular endoplasmic reticulum

The rough endoplasmic reticulum contains ribosome attached to the cytoplasmic side of the membrane and it forms a lace like system. The smooth Endoplasmic reticulum lacks the attached ribosome and it forms tubular structures.



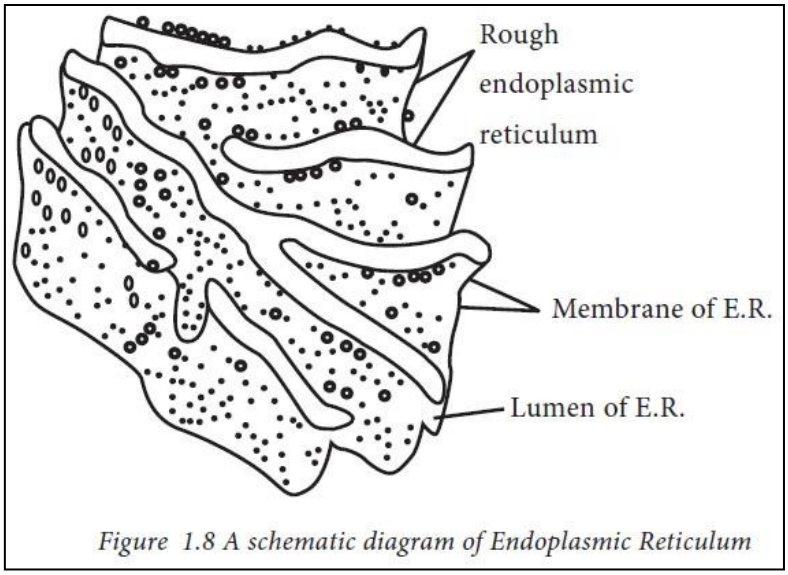


Figure 1.8 A schematic diagram of Endoplasmic Reticulum

endoplasmic reticulum (ER), in biology, a continuous membrane system that forms a series of flattened sacs within the cytoplasm of eukaryotic cells and serves multiple functions, being important particularly in the synthesis, folding, modification, and transport of proteins . All eukaryotic cells contain an endoplasmic reticulum (ER). In animal cells, the ER usually constitutes more than half of the membranous content of the cell. Differences in certain physical and functional characteristics distinguish the two types of ER, known as rough ER and smooth ER.

Rough ER is named for its rough appearance, which is due to the ribosomes attached to its outer (cytoplasmic) surface. Rough ER lies immediately adjacent to the cell nucleus, and its membrane is continuous with the outer membrane of the nuclear envelope. The ribosomes on rough ER specialize in the synthesis of proteins that possess a signal sequence that directs them specifically to the ER for processing. (A number of other

proteins in a cell, including those destined for the nucleus and mitochondria, are targeted for synthesis on free ribosomes, or those not attached to the ER membrane; *see* the article ribosome.) Proteins synthesized by the rough ER have specific final destinations. Some proteins, for example, remain within the ER, whereas others are sent to the Golgi apparatus, which lies next to the ER. Proteins secreted from the Golgi apparatus are directed to lysosomes or to the cell membrane; still others are destined for secretion to the cell exterior. Proteins targeted for transport to the Golgi apparatus are transferred from ribosomes on rough ER into the rough ER lumen, which serves as the site of protein folding, modification, and assembly.

The proximity of the rough ER to the cell nucleus gives the ER unique control over protein processing. The rough ER is able to rapidly send signals to the nucleus when problems in protein synthesis and folding occur and thereby influences the overall rate of protein translation. When misfolded or unfolded proteins accumulate in the ER lumen, a signaling mechanism known as the unfolded protein response (UPR) is activated. The response is adaptive, such that UPR activation triggers reductions in protein synthesis and enhancements in ER protein-folding capacity and ER-associated protein degradation. If the adaptive response fails, cells are directed to undergo apoptosis (programmed cell death).

Smooth ER, by contrast, is not associated with ribosomes, and its functions differ. The smooth ER is involved in the synthesis of lipids,including cholesterol and phospholipids, which are used in the production of new cellular membrane. In certain cell types, smooth ER plays an important role in the synthesis of steroid hormones from cholesterol. In cells of the liver, it contributes to the detoxification of drugs and harmful chemicals. The sarcoplasmic reticulum is a specialized type of smooth ER that regulates the calcium ion concentration in the cytoplasm of striated muscle cells.

The highly convoluted and labyrinthine structure of the ER led to its description in 1945 as a “lace-like reticulum” by cell biologists Keith Porter, Albert Claude, and Ernest Fullman, who produced the first electron micrograph of a cell. In the late 1940s and early 1950s, Porter and colleagues Helen P. Thompson and Frances Kallman introduced the term *endoplasmic reticulum* to describe the organelle. Porter later worked with Romanian-born American cell biologist George E. Palade to elucidate key characteristics of the ER.

The major functions of Endoplasmic reticulum are:

• They play a vital role in the formation of the skeletal framework

• They provide the increased surface area for cellular reactions

• They help in the formation of nuclear membrane during cell division

• They play a vital role in the synthesis of proteins, lipids, glycogen and other steroids like cholesterol, progesterone, testosterone, etc.

• They are responsible for the secretion, synthesis, modification and transportation of proteins and other carbohydrates to another organelle, which includes lysosomes, Golgi apparatus, plasma membrane, etc.

4. Golgi Apparatus

Camillo Golgi (1898) had made the first report on the densely stained reticular structures near the nucleus. Hence these were later named Golgi bodies, attributed to him. They consist of many flat, disc-shaped sacs or cisternae of 0.5µm to 1.0µm diameter. These are stacked parallel to each other. Varied numbers of cisternae are present in a Golgi complex. The Golgi cisternae are concentrically arranged near the nucleus with distinct convex *cis* or the forming face and concave *trans* or the maturing face.

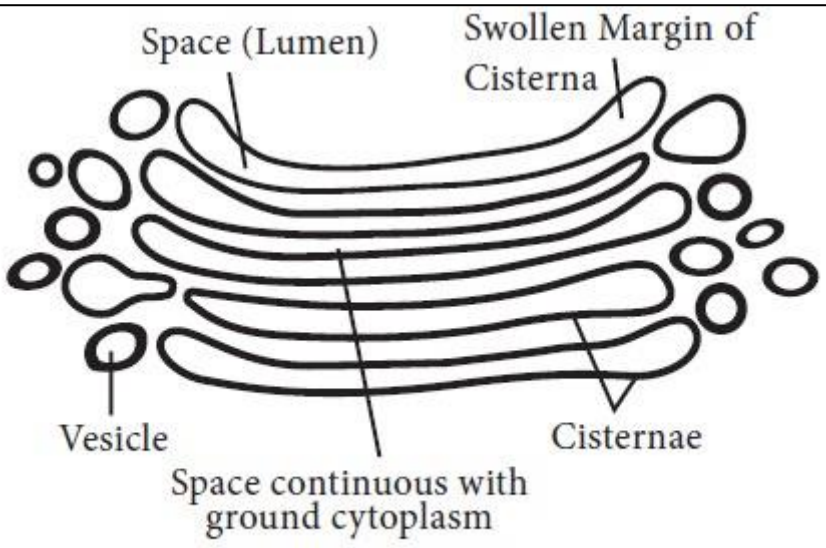


Figure 1.9.A schematic diagram of Golgi apparatus

The *cis* and the *trans* faces of the organelle are entirely different, but interconnected. The golgi apparatus principally performs the function of packaging materials, to be delivered either to the intra-cellular targets or secreted outside the cell. Materials to be packaged in the form of vesicles from the ER fuse with the *cis* face of the golgi apparatus and move towards the maturing face. This explains, why the golgi apparatus remains in close association with the endoplasmic reticulum. A number of proteins synthesized by ribosomes on the endoplasmic reticulum are modified in the cisternae of the golgi apparatus before they are released from its *trans* face. Golgi apparatus is the important site of formation of glycoproteins and glycolipids.

This is the Golgi apparatus from a pancreatic β cell as seen by transmission electron microscopy (TEM).

Pro-Insulin is synthesized on rough endoplasmic reticulum, transported to the Golgi apparatus by transfer vesicles, moves through the stack of flattened cisterna, and packaged into secretion granules.

Examine the morphology of the following structures:

- Golgi Apparatus (yellow) - stack of flattened cisterna.
- Transfer Vesicles (purple) - transfer newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus.
- Secretion Granules (gray) - the content of immature granules is homogenous, while mature granules have an electron-dense crystal of insulin (black). The mature granules are ~300 μm in diameter.

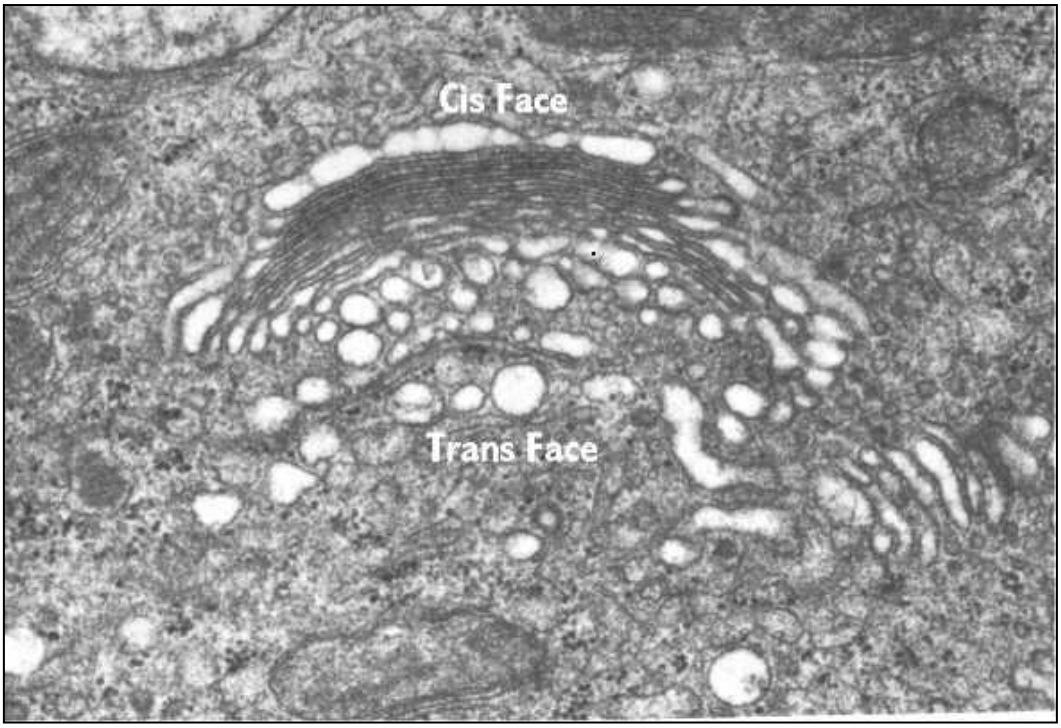


Fig: Electron microscopic structure of Golgi apparatus

Functions of Golgi apparatus

a. Golgi apparatus helps in protein sorting from one compartment to another by the secretory pathway.

b. Covalent modifications of proteins involving the addition of small sugar molecules occur in the ER and Golgi apparatus.

5. Ribosomes:

Ribosomes are the granular structures first observed under the electron microscope as dense particles by George Palade (1953). In the word ribosome, the pharse 'ribo' is derived from ribonucleic acid and 'somes' from the Greek word 'soma' which means 'body'. Ribosomes are tiny particles about 200 Å. They are composed of ribonucleic acid (RNA) and proteins. Ribosomes are not considered as organelles because of the lack of a membrane around them. However, when they are producing certain proteins they can become bound to the endoplasmic reticulum membrane. Free floating ribosomes are also present. Ribosomes are composed of both RNA and proteins. About 37 - 62% of ribosomes are made up of RNA and the rest is proteins. There are two types of ribosomes based on their sedimentation properties. Prokaryotes possess 70 S ribosomes and Eukaryotes possess 80 S ribosomes. The subunits of ribosomes are named owning to their sedimentation rate measured as special Svedberg Unit ('S'). The ribosomes share a core structure which is similar to all ribosomes despite differences in their size. The ribosomes are made up of two subunits - a small and a large subunit. The small subunit reads the mRNA while the large subunit joins the amino acids to form a chain of polypeptides.

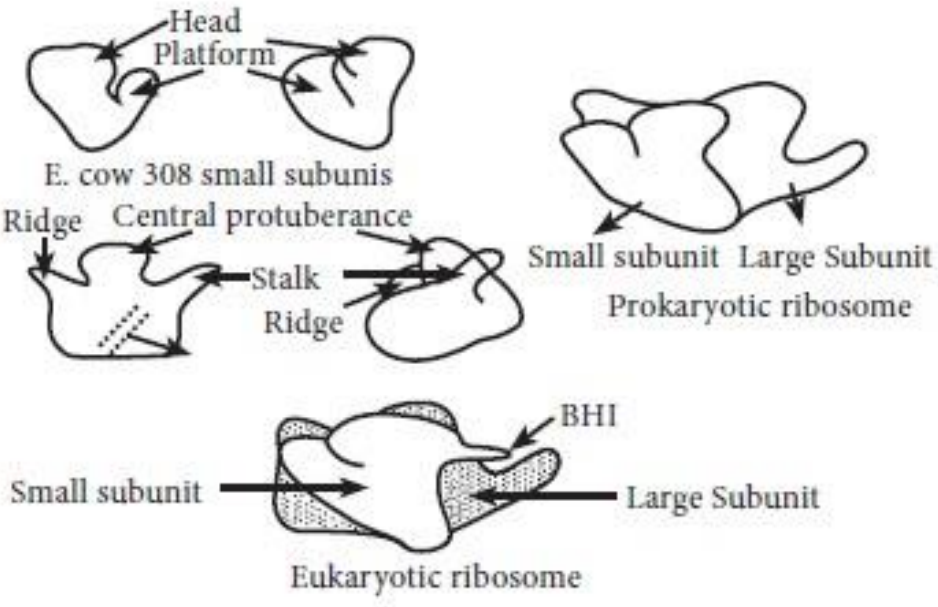


Figure 1.10 A schematic diagram of larger and smaller subunits of a ribosome

Functions of ribosomes:

· The bound and the free ribosomes are similar in structure and are involved in protein synthesis.

· The location of the ribosomes in a cell is a determining factor of the type of protein produced. If the ribosomes are free floating throughout the cell, the proteins that are used within the cell are produced. When ribosomes are attached to endoplasmic reticulum (referred as rough endoplasmic reticulum or rough ER), the proteins that are used inside the cell or outside the cell are produced.

6. Lysosomes

These are membrane bound vesicular structures formed by the process of packaging in the golgi apparatus. The isolated lysosomal vesicles have been found to be very rich in hydrolytic enzymes, called hydrolases such as lipases, proteases, carbohydrases, which are optimally active at the acidic pH. These enzymes are capable of digesting carbohydrates, proteins, lipids and nucleic acids.

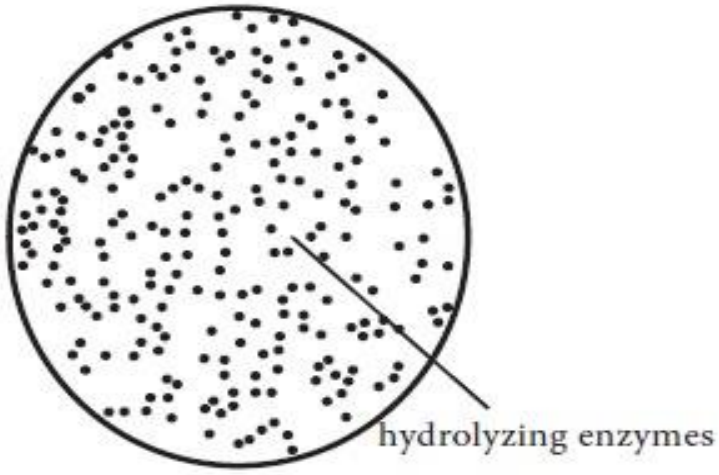


Figure 1.11 A schematic diagram of lysosome

7. Peroxisome:

Peroxisomes are microbodies that are abundantly present in mammalian liver and kidney, and also in plant cells. It depends on the type of eukaryotic cell. The matrix of Peroxisomes is rich in enzymes but a few enzymes are located in the membrane. The common enzymes present in the matrix of peroxisomes are catalases and peroxidases which metabolize a number of substrates. Enzymes present in the membrane of peroxisomes are cytochrome b5 and NADH cytochrome b5 reductase.

Functions of peroxisomes

· A major function of the peroxisome, in yeast and plant cells are to breakdown the fatty acid molecules, in a process called beta-oxidation. Peroxisomes are involved in lipid biosynthesis

· Peroxisomes contain enzymes required for the synthesis of plasmalogens

· Peroxisomes in seeds are responsible for the conversion of stored fatty acids to carbohydrates, which is critical in providing energy and raw materials for growth of the germinating plant.

Probable questions:

1. What do you mean by subcellular organelle?
2. Write down the name of three subcellular organellae with their function.
3. Describe the structure of mitochondria with diagram.
4. What are the functions of Peroxisome, ribosome and lysosomes?
5. Describe the structure of Ribosome with diagram.

Suggested readings:

1. Cooper G M, Hausman RE. 2009. The Cell: A Molecular Approach. V Ed. ASM Press and Sunderland
2. Snustad D P, Simmons MJ. 2009. Principles of Genetics. V Edition. John Wiley and Sons Inc
3. https://www.brainkart.com/article/Subcellular-organelles_34056/

UNIT VI

Radio labeling techniques: Detection and measurement of different types of radioisotopes normally used in biology, incorporation of radioisotopes in biological tissues and cells

Objective: In this unit we will discuss about Radio labeling techniques: Detection and measurement of different types of radioisotopes normally used in biology, incorporation of radioisotopes in biological tissues and cells.

Radioisotopes

Isotopes are atoms of an element with the same number of protons but different numbers of neutrons. Some of these isotopes are stable and exist fine with the extra neutrons. Others, however, are unstable, making these atoms radioactive. These are called radioisotopes and are useful in a variety of sciences, including biology, mining, industry and agriculture.

Carbon 14 dating

Carbon 14 is a radioactive isotope of carbon. Carbon normally has 12 neutrons, but isotopes with 13 and 14 neutrons also exists. Carbon with 14 neutrons is radioactive, decaying into nitrogen 14, but with a very long half-life (over 5,000 years). Therefore, in materials that were once alive, the amount of carbon 14 will steadily decrease over time. Calculating backwards, scientists can measure the amount of carbon 14 in a dead organism (say, a mammoth bone or wood from an ancient tree) and determine when the organism died, up to about 60,000 years ago.

Application of Radioisotope

I. Radioisotopes Used in Diagnostic Imaging (Tracers)

Radioisotopes can be used as tracers within a living organism to trace what is going on inside the organism at an atomic level; that is, radioisotopes can be injected or ingested by the organism, and researchers can trace the internal activities using the radioactivity. Medical specialists can use radioisotopes to see internal processes and conditions inside the human body. Gallium-67, for instance, can be ingested by patients. When the patient undergoes an MRI or PET scan, doctors can trace the substance inside the body and see what's going on without invasive surgery. Other radioisotopes, such as Iodine-123 and Iodine-125, help doctors diagnose thyroid disorders and metabolic disorders in a similar way.

II. Radioisotopes Used in Agriculture

Agriculturists can find out how effectively a plant uses a fertilizer by using certain radioisotopes. Phosphorus-32 is also used in molecular biology studies and genetics research. Sometimes, particular radioisotopes are used to cause a specific genetic mutation in plants to breed certain traits into or out of the plant. Some agriculturists are also able to use radioisotopes to cause genetic mutations in plants to make them less susceptible to attacks by pests.

III. Carbon dating

Radioactive carbon-14 decay could be used to estimate the age of organic materials. For example carbon dating revealed that the burial cloth of Jesus Christ originated during the medieval times between A.D. 1260-1390. Similarly mummified remains found frozen in the Italian Alps were at least 5000 years old

IV. Radionuclide Therapy (RNT)

This therapy makes use of radioisotopes that emits radiations upon their decay. These emitted radiations are used to target specific cancerous cells, tumours etc. to control their abnormal growth or completely eradicate it. For example cobalt-60 is use as a source of gamma radiation for radionuclide therapy, gamma knife radiosurgery and brachytherapy. Similarly targeted alpha therapy uses alphaemitting radionuclide such as Bi-213, Lead-212, and Boron-10 to for treating pancreatic, ovarian and melanoma cancers

V. Sterilizing

Sterilization of surgical instruments such as syringes, gloves, clothing and instruments using gamma mitting radionuclides including Cobalt-60, Cs-137 etc.

VI. Radiopharmaceuticals

Incorporation of radioisotopes to biologically active substances is introduced into body in order to observe the functioning of an organ functioning or a metabolic path way etc. For example Yttrium-90 and Iodine-131 is used as radiopharmaceuticals for the treatment of non-Hodgkin's lymphoma and hyperthyroidism respectively

- ## Half-life:

The number of atoms in a sample of radioisotope that disintegrate during a given time interval decreases logarithmically with time and is unaffected by chemical and physical factors that normally alter the rates of chemical processes (i.e., temperature, concentration, pressure, etc.). Radioactive decay is therefore a classic example of a first-order reaction. A convenient term used to describe the rate of decay of a radioisotope is the physical half-life, T_p. This is the amount of time required to reduce the amount of radioactive material to one-half its previous value.

Each radioactive isotope decays at a characteristic rate and therefore has a specific half-life (see Table 14-1). For example, the amount of radioactivity arising from a sample of ^{59}Fe is reduced to one-half its original value in 45.1 days, to one-fourth in 90.2 days, to one-eighth in 135.3 days, and so on. The amount of decay occurring in the course of a tracer experiment must be taken into account when radioisotopes of short physical half-life such as ^{24}Na, ^{32}P, ^{42}K, ^{59}Fe, ^{131}I, and ^{125}I are used. Of course, this is not a problem in tracer experiments employing ^{3}H and ^{14}C (Table 14-1) because the length of the experiment is insignificant in comparison with the half-life.

When radioisotopes are used in vivo experiments of extended duration, the turnover rate of the element in the body (or in the cell) must also be considered, for the rate of decrease of radioactivity will be a function in both radioactive decay and metabolic turnover. In these instances, a more useful term is the effective half-life, T_e, which is the amount of time required to reduce the radioisotope content of the body (or cell) to one-half its original value by the combined effects of decay and turnover; it is determined using the relationship

Where

T_e = effective half-life,

T_p = physical half-life, and

T_b = biological half-life and is defined as the normal amount of time required for the turnover of one-half of the body content of a given element (radioactive or nonradioactive).

Detection and measurement of different types of radioisotopes normally used in biology

The most widely used instrument for the detection and measurement of radiation is the Geiger-Muller (or G-M) counter.

The selection of instruments for the detection and measurement of radioisotopes is based primarily on the type and energy of the emitted radiation. The most commonly used detectors are: (1) Geiger- Muller counters, which are employed primarily with isotopes emitting beta particles of intermediate or high energy (E_{max} above 0.2 MeV) and which may also be used at low efficiency for the measurement of gamma radiation; (2) solid scintillation counters, which are generally employed with gamma ray- emitting isotopes; and (3) liquid scintillation counters, which are used with isotopes emitting low- energy beta particles (E_{max} below 0.2 MeV).

Geiger-Muller Counters:

The most widely used instrument for the detection and measurement of radiation is the Geiger-Muller (or G-M) counter. The detector itself, called a G-M tube, consists of a cylinder several inches long containing two electrodes and filled with a readily ionizable inert gas such as helium or argon.

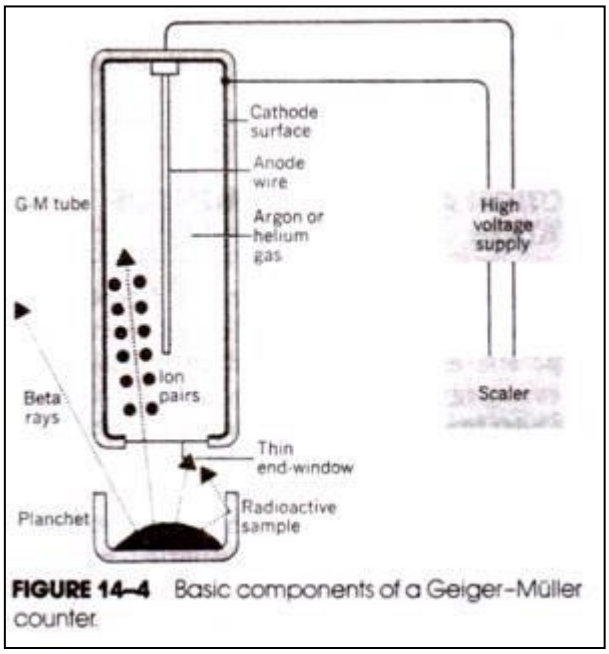


FIGURE 14–4 Basic components of a Geiger–Müller counter.

The insulated metallic internal surface of the cylinder serves as the cathode and a narrow wire passing down the center of the tube serves as the anode (Fig. 14-4). One end of the G-M tube is covered by a thin material such as mylar plastic or mica and is called the end-window. The anode and cathode terminals at the other end of the tube are connected to a source of high voltage and a scaler, a device that simply counts electrical pulses.

When a radioactive sample (usually deposited on a small metal disk called a planchet) is placed near the end-window, radiation enters the G-M tube, ionizing some of the gas molecules and forming a number of ion pairs (i.e., positively charged argon or helium atoms and electrons). If a sufficiently high electrical potential is applied to the electrodes, the ion pairs will migrate toward the appropriate electrode. During this migration, the ions collide with and ionize additional gas molecules, so that the passage of a single beta particle through the gas results in a large number of ions being collected at the electrodes.

These events produce an electrical pulse that is recorded by the scaler as a count. Ideally, each ionizing ray entering the G-M tube is registered as a count and the amount of radioactivity is expressed as counts per minute (cpm). Because radiation is emitted in all directions from a radioactive source, it is apparent that only a small percentage of the rays arising from the sample are directed toward the end-window. Therefore, even if all

the rays entering the G-M tube are detected and counted, the cpm recorded for the sample is only a fraction of the true rate of disintegration (i.e., disintegrations per minute, dpm) of the isotope.

This does not pose a serious problem when the relative isotope contents of a number of samples are to be determined (this is generally the case) and if constant geometric conditions are maintained for each sample (i.e., distance of the sample from the end-window, volume of sample, etc.). For some radioisotopes such as ^{3}H, ^{14}C; ^{35}S, and others of low E_{max}, much or all of the energy of the emitted beta particles may be dissipated before the ray enters the ionizing gas. For example, the energy may be expended within the sample itself (called self- absorption), in the air between the sample and the end-window, or in the material of the end-window.

Even with radioisotopes emitting beta particles of high E_{max} (Table 14-2), beta particles in the low region of the energy spectrum may go undetected. Because of the low specific ionization of gamma rays and the low density of the gas in the G-M tube, gamma rays may pass through the tube without causing ionizations and therefore go undetected. For these reasons, Geiger-Muller counters are usually not suitable for the detection and measurement of radioisotopes emitting gamma rays or beta particles of low E_{max}.

Even when no radioactive sample is placed below the end-window of the G-M tube, a small count is recorded. This is known as the background count and results from cosmic radiation (primarily gamma rays), naturally occurring radioisotopes in laboratory materials (such as ^{40}K in glass and naturally occurring ^{14}C and ^{3}H in organic compounds), radioactive samples left in the vicinity of the detector and electronic "noise" within the components of the counting system.

Therefore, the background count must always be subtracted from the count obtained for a radioactive sample. The magnitude of the background count may be reduced by placing lead shielding around the detector so that much of the cosmic radiation and radiations from other sources are absorbed before reaching the detector.

The total amount of radioisotope present in a sample at any instant in time may be determined from its rate of disintegration, that is, its dpm; the basic unit of measurement is the curie (Ci) and is defined as that quantity of radioisotope undergoing 2.22×10^{12}dpm. (Note that the curie content of a radioactive sample decreases exponentially with time at a rate determined by the physical half-life of the radioisotope.)

In most tracer experiments, the quantity of radioisotope used is generally at the millicurie (mCi) or microcurie (μCi) level. The curie content of a labeled compound is generally provided at the time of purchase so that the efficiency of the counting system may be determined by comparing the recorded cpm of an aliquot of the isotope with its known dpm.

Generally, this value is 10% or less for G-M counters but is much higher in solid and liquid scintillation systems. Once the efficiency of the counting system is known, then

the specific activity of a radioactive sample (which we may now define as the number of curies per unit mass of element or compound) collected during the course of a tracer experiment may be calculated from its observed counting rate and composition. G-M counters are effectively employed in tracer experiments involving ^{24}Na, ^{32}P, $^{36}C1$, and other "hard beta" emitters but generally are not used with ^{3}H and ^{14}C, which emit "soft beta" rays.

Solid Scintillation Counters:

Solid scintillation counters are used to detect and measure radioisotopes emitting gamma rays. The detector (Fig. 14-5) consists of a large crystal of thallium-activated sodium iodide and a photomultiplier tube encased in aluminum housing; the latter is interfaced with a preamplifier, a source of high voltage, and a scaler.

The radioactive sample to be analyzed is placed either against the end of the detector containing the crystal or, for greatly improved counting efficiency, into a well-shaped opening drilled into the crystal's surface (Fig. 14-5).

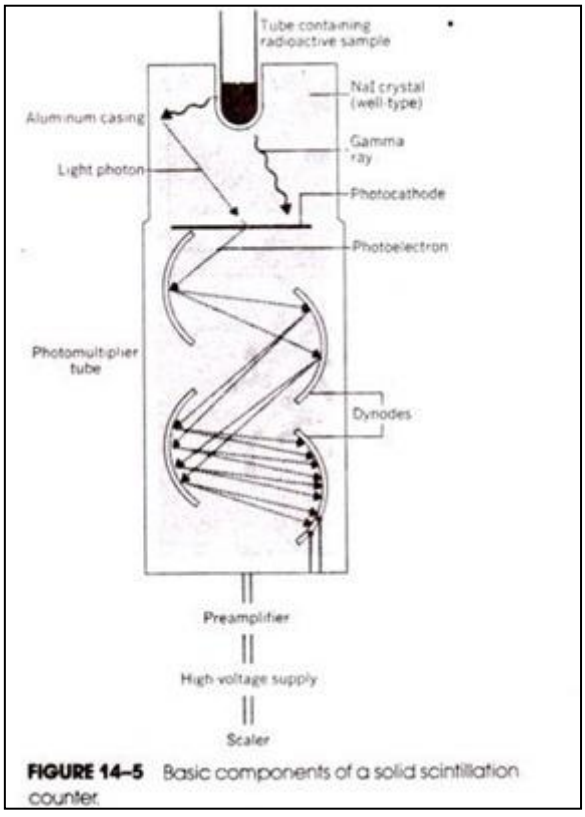


FIGURE 14–5 Basic components of a solid scintillation counter.

Because of its high density, the crystal absorbs much of the energy of the gamma rays, causing excitation of electrons of atoms composing the crystal and raising them to higher energy orbitals. As these electrons return again to their lower energy orbitals, flashes of light or scintillations are emitted and these are proportional in number to the number and energy of the gamma rays exciting the crystal.

The light photons are converted by the photomultiplier tube into electrical pulses of corresponding magnitude and frequency and these are relayed to the scaler. Because the magnitude of the electrical pulses produced is proportional to the energy of the gamma rays, and because gamma rays are mono- energetic, the inclusion of the appropriate circuitry in the counting system (i.e., a pulse-height analyzer) allows different gamma ray-emitting isotopes to be distinguished.

In contrast to G-M counters, few or no problems involving self-absorption and end-window absorption are incurred when solid scintillation methods are used with gamma ray-emitting isotopes. However, the use of constant geometry and lead shielding around the detector to reduce the magnitude of the background count is important.

- incorporation of radioisotopes in biological tissues and cells

Incorporation

- After inhalation, ingestion, or wound contamination, small radioisotope particles may be transported via blood or lymphatics into cells, tissues, and organs.
- Isotopes can be alpha-, beta-, or gamma-emitting.
- Radioisotopes can be incorporated into one or more organs specific for that isotope, (e.g. thyroid, lungs, kidneys, bones/bone marrow, or liver/spleen) resulting in exposure at that site.
- Medical countermeasures called decorporation agents or other procedures (e.g., diuresis) may be needed to remove radioisotopes that have been incorporated into tissues.
- Toxic effects of radioisotopes may be due to their chemical and/or radiological properties.
- **How to diagnose**
    - If after external decontamination, an appropriate radiation survey meter continues to identify significant residual radioactivity, suspect internal contamination.
    - Swab each nostril separately to help estimate level of internal (lung) contamination
    - Collect ≥70 mL spot urine sample for isotope measurement
        - Instructions for sample collection, labeling, packaging and shipping (HHS/CDC)
    - Consider total body radiation survey with modified hospital nuclear medicine equipment

Probable questions:

1. What is Radioisotope?
2. What is carbon dating?
3. Name the radioisotopes used in biological processes.
4. Discuss the application of Radioisotopes?
5. What is half life?
6. Write down the Process of incorporation of radioisotopes in biological tissues and cells
7. Describe Geiger-Muller (or G-M) counter with diagram. What is the application of Geiger-Muller (or G-M) counter?
8. Write short notes on **Solid Scintillation Counters.**

Suggested readings:

1. Goh, KM (1991) Carbon dating. Carbon Isotope Techniques 1: 125.
2. Wagner HN,Wiseman GA, Marcus CS (2002) guidelines for Radio immunotherapy for non-Hodgkin's lymphoma with 90Y-labeled anti-CD20 monoclonal antibody. J Nucl Med 43: 267-272.
3. Bakri A, Mehta K, Lance DR (2005) Sterilizing insects with ionizing radiation. In Sterile Insect Technique, Springer Netherlands, pp: 233-268.
4. Hosain Fazle (1996) Radiopharmaceuticals: therapeutic applications. 100 Years of X-rays and Radioactivity Bhabha Atomic Research Centre, Mumbai, India, pp: 447-461.
5. https://remm.hhs.gov/contamimage_6.htm#:~:text=Isotopes%20can%20be%20alpha%2D%2C%20beta,in%20exposure%20at%20that%20site.

UNIT VII

Molecular imaging of radioactive material, PET scan, safety guidelines

Objective:

In this unit we will discuss about Molecular imaging of radioactive material, PET scan, safety guidelines.

- **What are molecular imaging and nuclear medicine?**

Molecular imaging is a type of medical imaging that provides detailed pictures of what is happening inside the body at the molecular and cellular level. Where other diagnostic imaging procedures—such as x-rays, computed tomography (CT) and ultrasound—offer pictures of physical structure, molecular imaging allows physicians to see how the body is functioning and to measure its chemical and biological processes.

Molecular imaging includes the field of nuclear medicine, which uses very small amounts of radioactive materials (radiopharmaceuticals) to diagnose and treat disease.

Molecular imaging offers unique insights into the human body that enable physicians to personalize patient care. In terms of diagnosis, molecular imaging is able to:

- ✓ provide information that is unattainable with other imaging technologies or that would require more invasive procedures such as biopsy or surgery
- ✓ identify disease in its earliest stages and determine the exact location of a tumor, often before symptoms occur or abnormalities can be detected with other diagnostic tests

- **How do they help patients?**

As a tool for evaluating and managing the care of patients, molecular imaging studies help physicians:

- ✓ determine the extent or severity of the disease, including whether it has spread elsewhere in the body
- ✓ select the most effective therapy based on the unique biologic characteristics of the patient and the molecular properties of a tumor or other disease
- ✓ determine a patient's response to specific drugs
- ✓ accurately assess the effectiveness of a treatment regimen
- ✓ adapt treatment plans quickly in response to changes in cellular activity

- ✓ assess disease progression
- ✓ identify recurrence of disease and help manage ongoing care

- **When are they used?**

Molecular imaging procedures—which are noninvasive, safe and painless—are used to diagnose and manage the treatment of cancer, heart disease, brain disorders such as Alzheimer's and Parkinson's disease, gastrointestinal disorders, lung disorders, bone disorders, kidney and thyroid disorders, and more.

PET Scan

The positron emission tomography (PET) scan creates computerized images of chemical changes, such as sugar metabolism, that take place in tissue. Typically, the patient is given an injection of a substance that consists of a combination of a sugar and a small amount of radioactively labeled sugar. The radioactive sugar can help in locating a tumor, because cancer cells take up or absorb sugar more avidly than other tissues in the body.

After receiving the radioactive sugar, the patient lies still for about 60 minutes while the radioactively labeled sugar circulates throughout the body. If a tumor is present, the radioactive sugar will accumulate in the tumor. The patient then lies on a table, which gradually moves through the PET scanner incrementally several times during a 15-60-minute period. The PET scanner is used to detect the distribution of the sugar in the tumor and in the body. By the combined matching of a CT scan with PET images, there is an improved capacity to discriminate normal from abnormal tissues. A computer translates this information into the images that are interpreted by a radiologist.

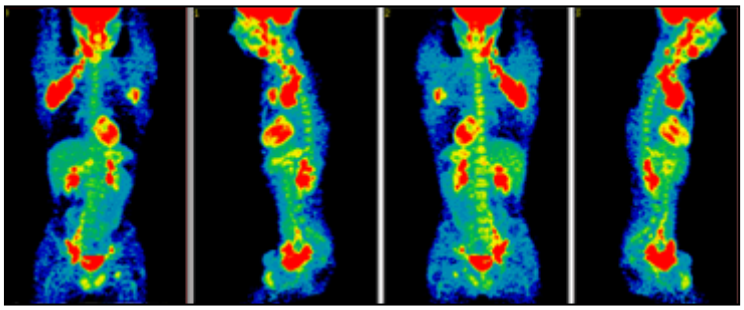


Fig: PET scans. Uptake of tracer in the lymph nodes involved with lymphoma in the groin, both axilla, and neck (red areas). Image coutesy of Dr. Jorge Carrasquillo, Nuclear Medicine Department, Clinical Center, National Institutes of Health.

PET scans may play a role in determining whether a mass is cancerous. However, PET scans are more accurate in detecting larger and more aggressive tumors than they are in locating tumors that are smaller than 8 mm a pinky nail (or half of a thumb nail)

and/or less aggressive cancers. The size of smallest tumor mass that can be found at PET is constantly improving. They may also detect cancer when other imaging techniques show normal results. PET scans may be helpful in evaluating and staging recurrent disease (cancer that has come back). PET scans are beginning to be also commonly used to check if a treatment is working - if a tumor cells are dying and thus using less sugar.

SPECT Scan

Similar to PET, single photon emission computed tomography (SPECT) uses radioactive tracers and a scanner to record data that a computer constructs into two- or three-dimensional images. A small amount of a radioactive drug is injected into a vein and a scanner is used to make detailed images of areas inside the body where the radioactive material is taken up by the cells. SPECT can give information about blood flow to tissues and chemical reactions (metabolism) in the body.

In this procedure, antibodies (proteins that recognize and stick to tumor cells) can be linked to a radioactive substance. If a tumor is present, the antibodies will stick to it. Then a SPECT scan can be done to detect the radioactive substance and reveal where the tumor is located.

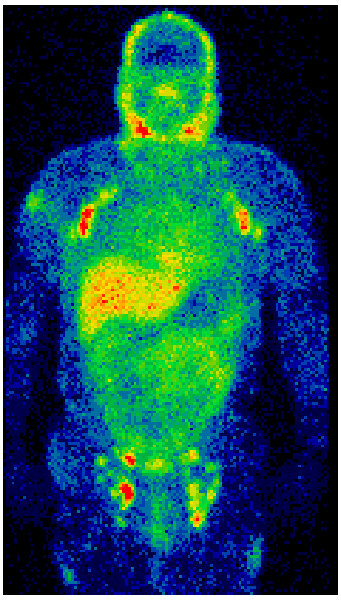


Fig: SPECT scan. High levels of antibody in pelvis and axilla (red) and uptake in skin of the thigh and right shoulder (green) showing areas of cutaneous T-cell lymphoma. Image courtesy of Dr. Jorge Carrasquillo, Nuclear Medicine Department, Clinical Center.

- **Rules for handling radioisotopes**

Rules for handling radioisotopes are listed below

1. Wear protective clothing, gloves and glasses.
2. Use smallest amount possible.
3. Keep materials safe, secure and well labeled.
4. Monitor working area frequently.
5. Avoid foods and drinks in laboratory.
6. Wash and monitor hands after experiment.
7. Follows all local rules such as for the dispensing of stock and the disposal of waste.
8. Do not create radioactive aerosols or dust and for penetrating radiations (32P).
9. Maximise the distance between source and you.
10. Minimise the time of exposure.
11. Maintain shielding at all times.

Probable questions:

1. Define Molecular imaging.
2. How molecular imaging and nuclear medicine help patients.
3. What is PET Scan?
4. Write down the rules of Rules for handling radioisotopes.

Suggested readings:

1. https://www.snmmi.org/AboutSNMMI/Content.aspx?ItemNumber=6433#:~:text=Molecular%20imaging%20includes%20the%20field,to%20diagnose%20and%20treat%20disease
2. https://s3.amazonaws.com/rdcms-snmmi/files/production/public/FileDownloads/Patients/FactSheets/What%20is%20Nuclear%20Medicine%20and%20Molecular%20Imaging.pdf

UNIT VIII

Chromatography: TLC, Column chromatography, Affinity chromatography

Objective:

In this unit we will discuss about Chromatography: TLC, Column chromatography, Affinity chromatography

What is chromatography?

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- The Russian botanist Mikhail Tswett coined the term chromatography in 1906.
- The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of **fatty acid** mixtures.
- A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.
- It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.

Principle of Chromatography

- **Distribution of analytes between phases:**

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

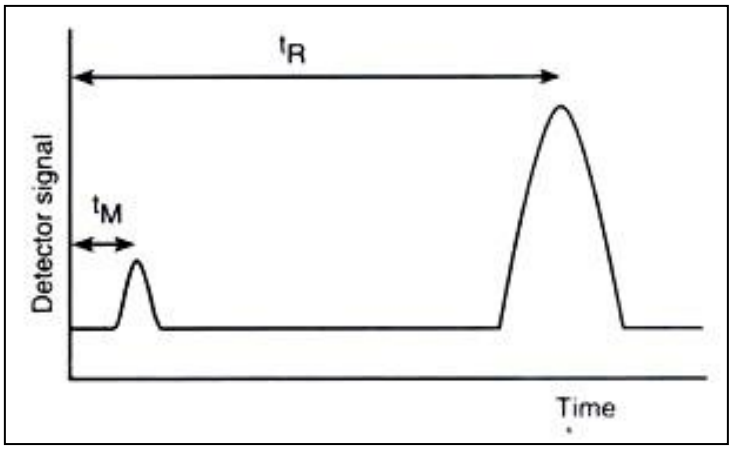
A mobile ↔ A stationary

The equilibrium constant, K, is termed the partition coefficient, defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase. The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the retention time (t_R). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called t_M.

A term called the retention factor, k', is often used to describe the migration rate of an analyte on a column. You may also find it called the capacity factor. The retention factor for analyte A is defined as

$$k_A' = (t_R - t_M)/t_M.$$

t_R and t_M are easily obtained from a chromatogram. When an analytes retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between one and five.



We define a quantity called the selectivity factor, α, which describes the separation of two species (A and B) on the column

$$\alpha = k_B' / k_A'$$

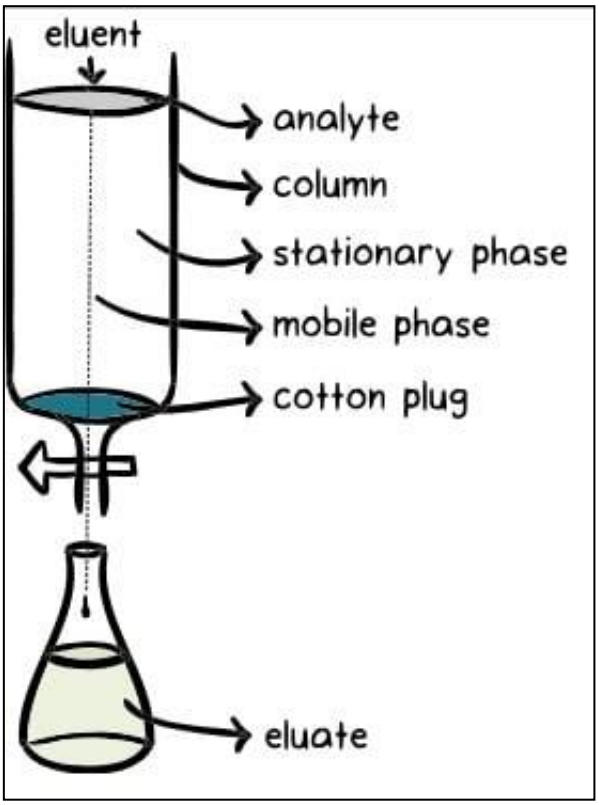


Image Source: Khan Academy

When calculating the selectivity factor, species A elutes faster than species B. The selectivity factor is always greater than one.

Three components thus form the basis of the chromatography technique.

1. **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
2. **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
3. **Separated molecules** The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.

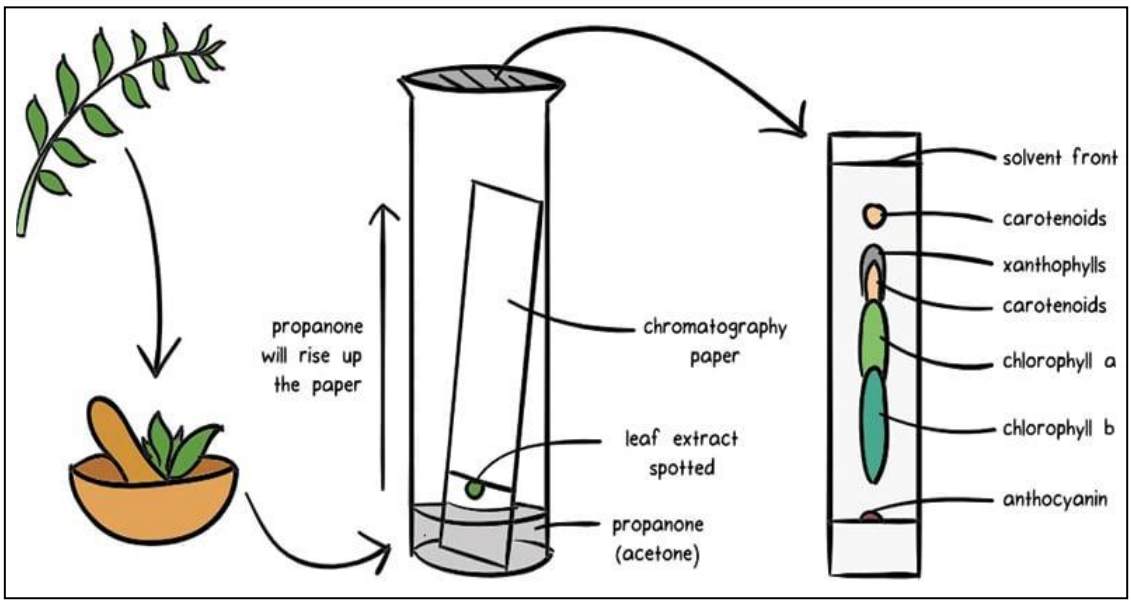


Image Source: Khan Academy

Various technical terminologies of use in the topic:

i. The analyte is the substance which is to be separated during chromatography.

ii. Analytical chromatography is used to determine the existence and possibly also the concentration of analyte(s) in a sample.

iii. A bonded phase is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

iv. A chromatogram is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

v. A chromatograph is equipment that enables a sophisticated separation, e.g., gas chromatographic or liquid chromatographic separation.

vi. The effluent is the mobile phase leaving the column.

vii. An immobilized phase is a stationary phase which is immobilized on the support particles, or on the inner wall of the column tubing.

viii. The mobile phase is the phase which moves in a definite direction. It may be a liquid (LC and CEC), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC).

ix. Preparative chromatography is used to nondestructively purify sufficient quantities of a substance for further use, rather than analysis.

x. The retention time is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

xi. The sample is the matter analysed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.

xii. The solute refers to the sample components in partition chromatography.

xiii. The solvent refers to any substance capable of solubilizing other substance, especially the liquid mobile phase in LC.

Applications of Chromatography

Pharmaceutical sector

- To identify and analyze samples for the presence of trace elements or chemicals.
- Separation of compounds based on their molecular weight and element composition.
- Detects the unknown compounds and purity of mixture.
- In drug development.

Chemical industry

- In testing water samples and also checks air quality.
- HPLC and GC are very much used for detecting various contaminants such as polychlorinated biphenyl (PCBs) in pesticides and oils.
- In various life sciences applications

Food Industry

- In food spoilage and additive detection
- Determining the nutritional quality of food

Forensic Science

- In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies

- Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.
- HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.

Thin Layer Chromatography (TLC)

What Is Thin Layer Chromatography?

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_f) expressed as:

R_f = dist. travelled by sample / dist. travelled by solvent

The factors affecting retardation factor are the solvent system, amount of material spotted, adsorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatography techniques.

Thin Layer Chromatography Principle

Like other chromatographic techniques, thin-layer chromatography (TLC) depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

Thin Layer Chromatography Diagram

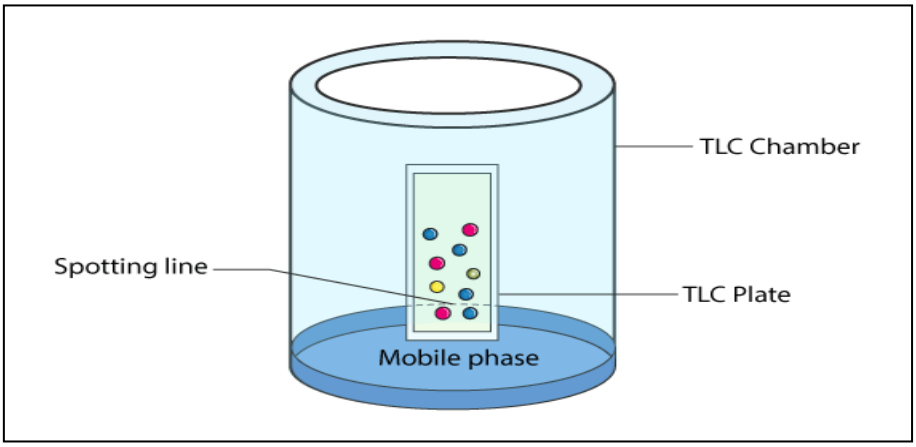


Diagram of Thin Layer Chromatography

Thin Layer Chromatography Procedure

Before starting with the Thin Layer Chromatography Experiment, let us understand the different components required to conduct the procedure along with the phases involved.

1. Thin Layer Chromatography Plates – ready-made plates are used which are chemically inert and stable. The stationary phase is applied on its surface in the form of a thin layer. The stationary phase on the plate has a fine particle size and also has a uniform thickness.
2. Thin Layer Chromatography Chamber – Chamber is used to develop plates. It is responsible to keep a steady environment inside which will help in developing spots. Also, it prevents the solvent evaporation and keeps the entire process dust-free.
3. Thin Layer Chromatography Mobile phase – Mobile phase is the one that moves and consists of a solvent mixture or a solvent. This phase should be particulate-free. The higher the quality of purity the development of spots is better.
4. Thin Layer Chromatography Filter Paper – It has to be placed inside the chamber. It is moistened in the mobile phase.

Thin Layer Chromatography Experiment

The stationary phase that is applied to the plate is made to dry and stabilize.

- To apply sample spots, thin marks are made at the bottom of the plate with the help of a pencil.
- Apply sample solutions to the marked spots.
- Pour the mobile phase into the TLC chamber and to maintain equal humidity, place a moistened filter paper in the mobile phase.

- Place the plate in the TLC chamber and close it with a lid. It is kept in such a way that the sample faces the mobile phase.
- Immerse the plate for development. Remember to keep the sample spots well above the level of the mobile phase. Do not immerse it in the solvent.
- Wait till the development of spots. Once the spots are developed, take out the plates and dry them. The sample spots can be observed under a UV light chamber.

Thin Layer Chromatography Applications

1. The qualitative testing of Various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.
2. TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.
3. Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc.
4. It is widely used in separating multicomponent pharmaceutical formulations.
5. It is used for the purification of samples and direct comparison is done between the sample and the authentic sample.
6. It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives
7. It is used in the cosmetic industry.
8. It is used to study if a reaction is complete.

Disadvantages of Thin Layer Chromatography:

1. Thin Layer Chromatography plates do not have longer stationary phase.
2. When compared to other chromatographic techniques the length of separation is limited.
3. The results generated from TLC are difficult to reproduce.
4. Since TLC operates as an open system, some factors such as humidity and temperature can be can affect the final outcome of the chromatogram.
5. The detection limit is high and therefore if you want a lower detection limit, you cannot use TLC.
6. It is only a qualitative analysis technique and not quantitative.

Column chromatography

What is Column Chromatography?

In chemistry, Column chromatography is a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid.

Column chromatography separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allows them to get separated in fractions. This technique can be used on a small scale as well as large scale to purify materials that can be used in future experiments. This method is a type of adsorption chromatography technique.

Column Chromatography Principle

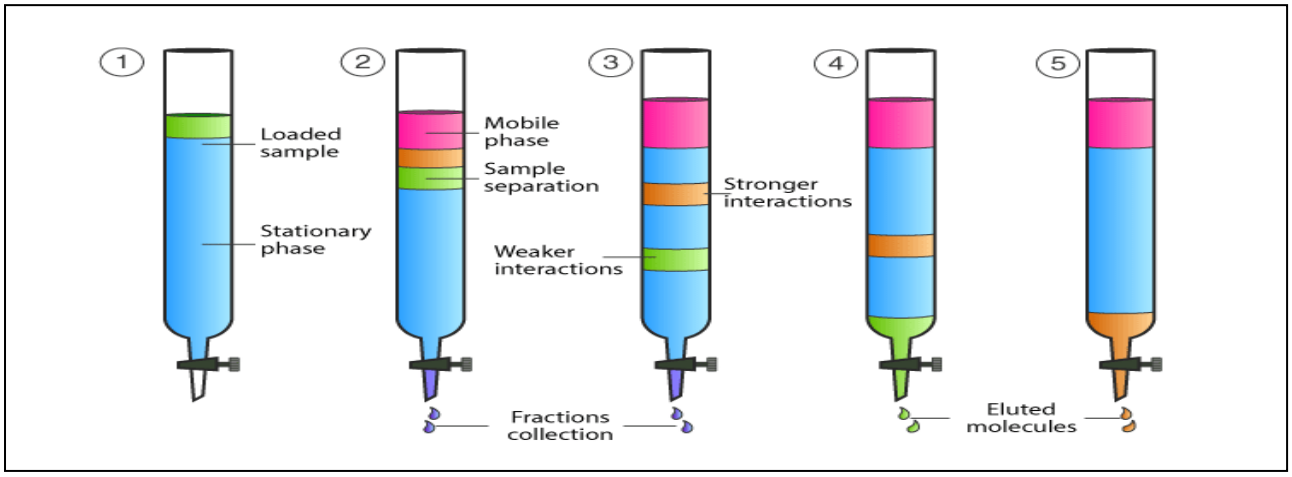
When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to the stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

R_f = the distance travelled by solute/ the distance travelled by the solvent

R_f is the retardation factor.

Column Chromatography Diagram



Column Chromatography Diagram

Elution

Elution is a chemical process that involves removing a material's ions by ion exchange with another material. The chromatographic technique of extracting an adsorbed substance from a solid adsorbing media using a solvent. The eluent is the solvent or mobile phase that passes through the column. When the polarity of the eluent matches the polarity of the molecules in the sample, the molecules desorb from the adsorbent and dissolve in the eluent.

The fraction of the mobile phase that transports the sample components is known as eluent. The mixture of solute and solvent that exits the column is known as an eluate. The eluate is made up of the mobile phase and analytes. A substance that separates and moves constituents of a mixture through the column of a chromatograph. The eluent in liquid chromatography is a liquid solvent whereas in gas chromatography is a carrier gas.

Column Chromatography Procedure

Before starting with the Column Chromatography Experiment let us understand the different phases involved.

Mobile phase – This phase is made up of solvents and it performs the following functions:

1. It acts as a solvent-sample mixture that can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample to form bands.
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column
4. Some examples of solvents used as mobile phases based on their polarity are – ethanol, acetone, water, acetic acid, pyridine, etc.
5. Stationary phase – It is a solid material which should have good adsorption properties and meet the conditions given below:
6. Shape and size of particle: Particles should have a uniform shape and size in the range of 60 – 200μ in diameter.
7. Stability and inertness of particles: high mechanical stability and chemically inert. Also, no reaction with acids or bases or any other solvents was used during the experiment.
8. It should be colourless, inexpensive and readily available.
9. Should allow free flow of mobile phase
10. It should be suitable for the separation of mixtures of various compounds.

Column Chromatography Experiment

- The stationary phase is made wet with the help of solvent as the upper level of the mobile phase and the stationary phase should match. The mobile phase or eluent is either solvent or a mixture of solvents. In the first step the compound mixture that needs to be separated, is added from the top of the column without disturbing the top level. The tap is turned on and the adsorption process on the surface of silica begins.
- Without disturbing the stationary phase solvent mixture is added slowly by touching the sides of the glass column. The solvent is added throughout the experiment as per the requirement.
- The tap is turned on to initiate the movement of compounds in the mixture. The movement is based on the polarity of molecules in the sample. The non-polar components move at a greater speed when compared to the polar components.
- For example, a compound mixture consists of three different compounds viz red, blue, green then their order based on polarity will be as follows blue>red>green
- As the polarity of the green compound is less, it will move first. When it arrives at the end of the column it is collected in a clean test tube. After this, the red compound is collected and at last blue compound is collected. All these are collected in separate test tubes.

Column Chromatography Applications

- Column Chromatography is used to isolate active ingredients.
- It is very helpful in separating compound mixtures.
- It is used to determine drug estimation from drug formulations.
- It is used to remove impurities.
- Used to isolate metabolites from biological fluids.

Types of Column Chromatography:

1. Adsorption column chromatography – Adsorption chromatography is a technique of separation, in which the components of the mixture are adsorbed on the surface of the adsorbent.

2. Partition column chromatography – The stationary phase, as well as mobile phase, are liquid in partition chromatography.

3. Gel column chromatography – In this method of chromatography, the separation takes place through a column packed with gel. The stationary phase is a solvent held in the gap of a solvent.

4. Ion exchange column chromatography – A chromatography technique in which the stationary phase is always ion exchange resin.

Affinity Chromatography

Affinity Chromatography:

It is a chromatographic method of separating biochemical mixtures, based on a highly specific biologic interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a stationary phase that reversibly binds to a known subset of molecules.

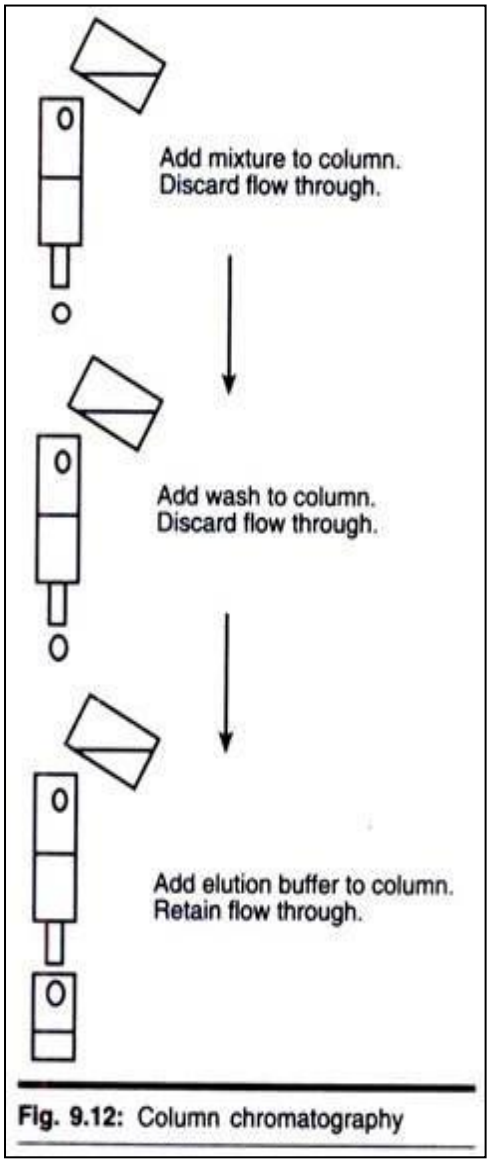


Fig. 9.12: Column chromatography

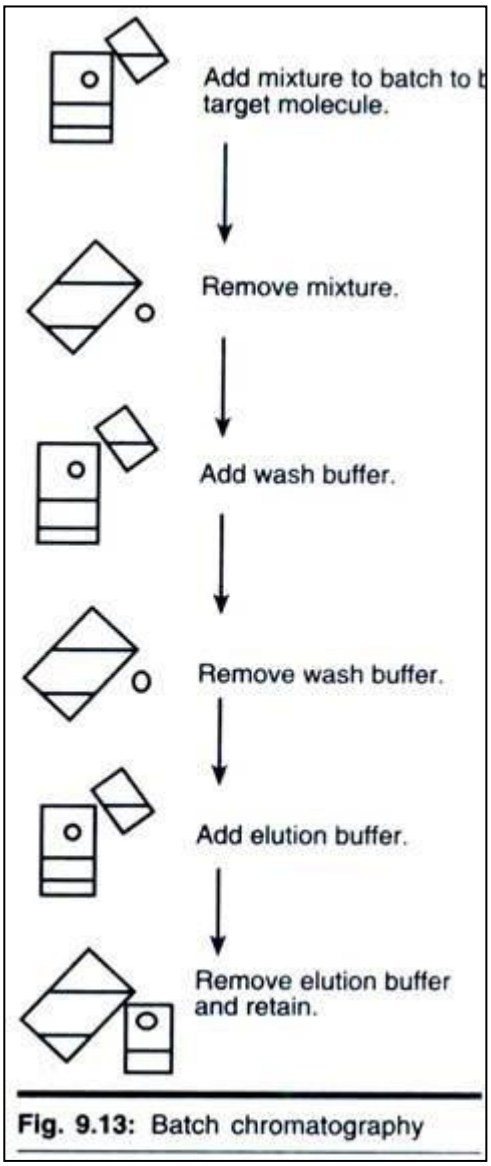


Fig. 9.13: Batch chromatography

Affinity chromatography can be used to:

i. Purify and concentrate a molecule from a mixture into a buffering solution.

ii. Reduce the amount of a molecule in a mixture.

iii. Discern what biological compounds bind to a particular molecule, such as drugs.

Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well known and defined property which can be exploited during the affinity purification process. The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium.

The other molecules in solution will not become trapped as they do not possess this property. The solid medium can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution.

Binding to the solid phase may be achieved by column chromatography, whereby the solid medium is packed onto a chromatography column, the initial mixture run through the column to allow binding, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure (as opposed to HPLC or FPLC).

Alternatively binding may be achieved using a batch treatment, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase (by centrifugation, for example), removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the eluate. Sometimes a hybrid method is employed, the binding is done by the batch method, then the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

A third method, expanded bed adsorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensures that the solid phase does not exit the column with the liquid phase.

Application:

Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts and antibody purification from blood serum.

1. Antibody affinity:

Another use of the procedure is the affinity purification of antibodies from blood serum. If serum is known to contain antibodies against a specific antigen (for example, if the serum comes from an organism immunized against the antigen concerned), then it can

be used for the affinity purification of that antigen. For example, if an organism is immunized against a GST-fusion protein it will produce antibodies against the fusion-protein, and possibly antibodies against the GST tag as well. The protein can then be covalently coupled to a solid support such as agarose.

For thoroughness the GST protein and the GST-fusion protein can each be coupled separately. The serum is initially allowed to bind to the GST affinity matrix. This will remove antibodies against the GST part of the fusion protein. The serum is then separated from the solid support and allowed to bind to the GST-fusion protein matrix. This allows any antibodies that recognize the antigen to be captured on the solid support.

Elution of the antibodies of interest is most often achieved using a low pH buffer such as glycine, pH 2.8. The eluate is collected into a neutral tris or phosphate buffer to neutralize the low pH elution buffer and halts any degradation of the antibody's activity. This is a nice example as affinity purification is used to purify the initial GST-fusion protein to remove the undesirable anti-GST antibodies from the serum and to purify the target antibody.

2. Immobilized metal ion affinity chromatography:

Immobilized metal ion affinity chromatography (IMAC) is based on the specific coordinate covalent binding of amino acids, allowing proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions, such as cobalt, nickel, copper, zinc, or iron ions. Many naturally occurring proteins do not have an affinity for metal ions; therefore, recombinant DNA techniques are used to introduce this property into a protein of interest.

Methods used to elute the protein of interest include changing the pH, or adding a specific molecule, such as imidazole. Similarly, the plant protein concanavalin A is able to bind with glucose immobilized on a column matrix. The concanavalin molecule is separated from the glucose by increasing the glucose concentration, displacing the concanavalin from the immobilized glucose.

3. Recombinant proteins:

Possibly the most common use of affinity chromatography is for the purification of recombinant proteins. Proteins with a known affinity are tagged in order to aid their purification. The protein may have been genetically modified so as to allow it to be selected for affinity binding; this is known as a fusion protein. Tags include His-tags and GST (glutathione-S-transferase) tags.

His_6-tags have an affinity for nickel or cobalt ions which are coordinated with NTA for the purposes of solid medium entrapment. For elution, an excess amount of a compound able to act as a nickel ligand, such as imidazole, is used. GST has an affinity for glutathione — commercially available immobilized as glutathione agarose. For elution, excess glutathione is used to displace the tagged protein.

Probable questions:

1. Define chromatography? What is the basic principle of chromatography?
2. Discuss the basic principle of thin layer chromatography.
3. Write down the application of TLC.
4. What is Column Chromatography?
5. What is elution?
6. What is stationary phase?
7. What is mobile phase?
8. Discuss the basic principle of Column chromatography.
9. Mention the application of Column chromatography
10. Mention the application of Affinity chromatography.

Suggested readings:

1. https://chromatography.conferenceseries.com/events-list/applications-of-chromatography
2. http://www.biologydiscussion.com/biochemistry/chromatography-techniques/top-12-types-of-chromatographic-techniques-biochemistry/12730
3. http://library.umac.mo/ebooks/b28050630.pdf
4. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5206469/
5. https://byjus.com/chemistry/thin-layer-chromatography/

UNIT IX

DNA sequencing method

Objective:

In this unit we will discuss about DNA sequencing method.

DNA Sequencing Definition

DNA sequencing is a method used to determine the precise order of the four nucleotide bases – adenine, guanine, cytosine and thymine - that make up a strand of DNA. These bases provide the underlying genetic basis (the genotype) for telling a cell what to do, where to go and what kind of cell to become (the phenotype). Every organism's DNA consists of a unique sequence of nucleotides. Determining the sequence can help scientists compare DNA between organisms, which can help show how the organisms are related.

The following methods highlight the top three methods used in gene sequencing. The methods are:

1. Maxam & Gilbert's Chemical Degradation Method

2. Sanger and Coulson's Dideoxynucleotide Synthetic Method

3. Direct DNA sequencing using PCR.

1. Maxam & Gilbert's Chemical Degradation Method:

In this method the following steps are involved (Fig. 1)

i. The 3′ ends of DNA fragments are labelled.

ii. The labelled strands are then separated, of which both strands are labelled at 3′ ends.

iii. The mixture is divided into four samples, each treated with a different reagent having the property of destroying either only G or only C or A and G, T and C; the concentration is adjusted in such a way that 50% of target base is destroyed.

iv. Fragments of different sizes having ^{32}P are produced.

v. Electrophoresis is done using each of the four samples in four different lanes of the gel.

vi. Autoradiograph of the gel helps to determine the sequence from the position of bands in four lanes.

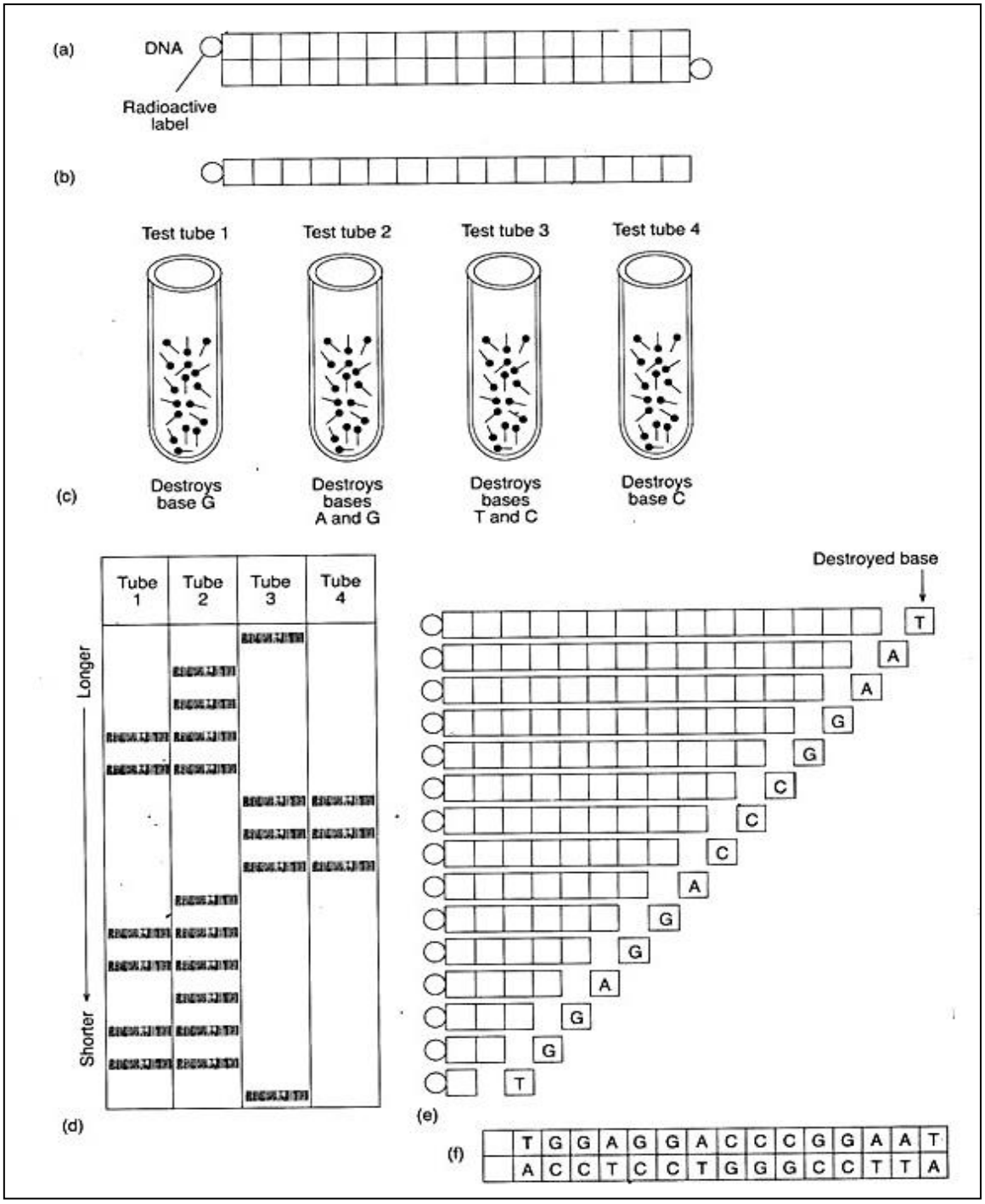


Fig 1: Different steps involved in Maxam & Gilbert's Chemical Degradation Method of DNA sequencing

2. Sanger's Method:

The first DNA sequencing method devised by Sanger and Coulson in 1975 was called plus and minus sequencing that utilized *E. coli* DNA pol I and DNA polymerase from bacteriophage T_4 with different limiting triphosphates. This Sanger and Gilbert methods of sequencing DNA are often called "first-generation" sequencing because they were the

first to be developed. This technique had a low efficiency. Sanger and co-worker (1977) eventually invented a new method for DNA sequencing via enzymatic polymerization that basically revolutionized DNA sequencing technology.

The Procedure:

The DNA to be sequenced is prepared as a single strand (Fig. 2).

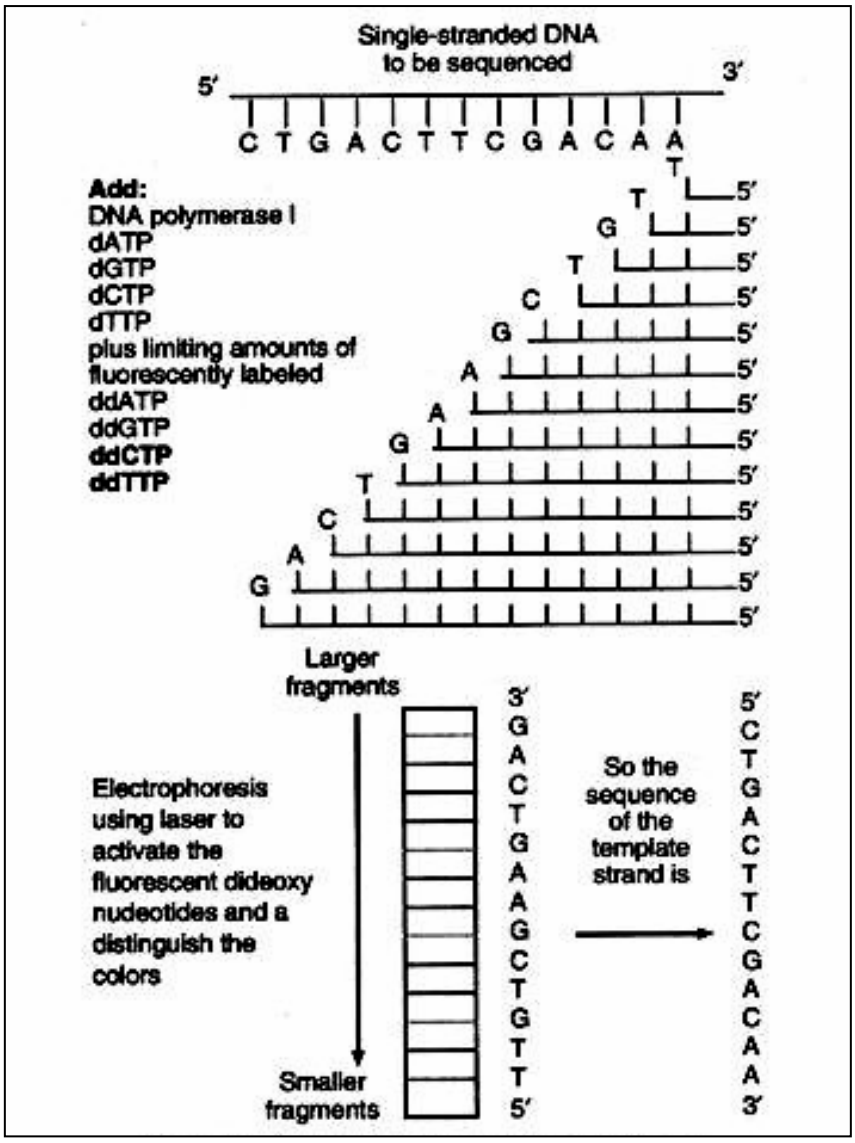


Fig 2: Sanger's method of DNA sequencing

This template DNA is mixed with the following:

(a) A mixture of all four normal (deoxy) nucleotides in sample quantities

i. dATP ii. dGTP iii. dCTP iv. dTTP

(b) A mixture of all four dideoxynucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different colour:

i. ddATP ii. ddGTP iii. ddCTP iv. ddTTP

(c) DNA polymerase I:

Because all four normal nucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxy nucleotide instead of the normal deoxynucleotide. If the ratio of normal nucleotide to the dideoxy versions is high enough, some DNA strands will succeed in adding several hundred nucleotides before insertion of the dideoxy version halts the process.

At the end of the incubation period, the fragments are separated by length from longest to shortest. The resolution is so good that a difference of one nucleotide is enough to separate that strand from the next shorter and next longer strand. Each of the four dideoxynucleotides fluorescence a different colour when illuminated by a laser beam and an automatic scanner provides a printout of the sequence.

The method involves the following steps:

i. Four reaction tubes are set up each containing single stranded DNA sample to be sequenced, ail four dNTPs (radioactively labelled) and an enzyme for DNA synthesis (DNA polymerase I).

ii. Each tube also contains a small amount of (much smaller amount relative to four dNTPs) one of the ddNTP, so that four tubes have each different ddNTP, bringing about termination at a specific base – Adenine (A), Cytosine (C), Thymine (T), and Guanine (G).

iii. The fragments generated by random incorporation of ddNTP leads to termination of reaction and so the different fragments are produced which can be separated by high resolution polyacrylamide gel, four adjoining lanes are loaded by four different samples.

iv. The gel is then auto-radiographed, the position of different bands in each lane can be visualized, and based on the position of the bands, the DNA sequence can be read out very easily.

3. Direct DNA Sequencing using PCR:

PCR is also used for sequencing the amplified DNA product. This method is more reliable, less time consuming and can utilize either the whole genomic DNA or cloned fragments for sequencing a particular DNA segment.

The sequencing in this method involves only two steps:

i. Generation of sequencing templates (double stranded or single stranded using PCR).

ii. Sequencing of PCR products either with the thermolabile DNA polymerase or with the thermo-stable Taq DNA polymerase.

So, here the DNA sequencing does not require any cloning vector. In both the cases of enzyme, Sanger's dideoxy method or Maxam- Gilbert's chemical method can be used. In Sanger's method, as usual, the sequencing primer is labelled with ^{32}P and the mixtures

with amplified DNA, Taq polymerase and appropriate buffer are incubated at 70°C for 5 min.

The reaction is stopped by addition of formamide and mixtures are allowed to run polyacrylamide sequencing gel, which can be read by computer or manually.

Genome Projects:

The technology for large- scale DNA sequencing has enable scientists to undertake genome sequencing project in a realistic time scale. Since the time of first 'large' genome sequencing in bacteriophage X in 1983, the projects on different groups have been completed.

Some notable examples include the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisae*, the weed Arabidopsis thaliana, the rice *Oryza sativa*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the mouse *Mus musculus*, the primate chimpanzee *Pan troglodytes*, the human *Homo sapiens sapiens*.

DNA Sequencing Applications

There are various applications of DNA sequencing in different fields, including:

- DNA sequencing has helped to obtain detailed information about the genetic makeup of living organisms. It has also helped to discover new genes and understand their function.
- It can be used as a diagnostic tool to identify genetic variations associated with diseases, which helps in the development of new therapeutic tools.
- It can also be used in evolutionary studies to study the relationship between different species and understand evolutionary processes.
- It can be used in environmental studies to study the diversity of life which aids in better understanding and protecting biodiversity.
- It can also be used in criminal investigations to analyze DNA evidence and identify suspects.
- It is a fundamental aspect of biological research and is utilized in diverse fields such as biotechnology, virology, forensic biology, and medical diagnostics.

Probable questions:

1. Define DNA sequencing.
2. Describe the Sanger and Coulson's Dideoxynucleotide Synthetic Method of DNA sequencing.
3. Describe the Direct DNA Sequencing using PCR.
4. What is genome project?
5. Write down the application of DNA sequencing.

Suggested readings:

1. Snustad D P, Simmons MJ. 2009. Principles of Genetics. V Edition. John Wiley and Sons Inc
2. Strickberger M. W – Genetics; Macmillam
3. Tamarin R. H. – Principles of Genetics; McGraw Hill
4. Klug W S, Cummings MR, Spencer CA. 2012. Concepts of Genetics. Xth Ed. Benjamin Cummings

UNIT X

Comet assay

Objective: In this unit we will discuss about Comet assay.

Comet Assay

The Comet Assay, also known as single cell gel electrophoresis (SCRE), helps scientists to determine whether there has been deoxyribonucleic acid (DNA) damage to a single cell from apoptosis (cell death) or cytotoxicity (toxicity to cells) and the extent of this damage. The DNA of an organism encodes the genetic instructions which govern the structure and functions of the constituent cells.

Damage is defined as single or double-stranded breaks in the DNA – such damage can be caused by chemicals or UV radiation. The Comet Assay can be applied to both eukaryotic (cell with a membrane-bound nucleus with the DNA structured in chromosomes) and prokaryotic (a cell with neither a membrane-bound nucleus nor DNA structured in chromosomes) cells.

The Comet Assay is used in oncology to screen for genotoxicity (damage to genetic materials), gauge chemoprevention effectiveness, and determine the effectiveness of specific agents to halt progression to invasive cancer in the human body.

- **Comet definition**

The comet definition is determined by working out the percentage of DNA in the tail, dividing this by the integrated tail intensity and multiplying this by 100. The figure achieved is then divided by the total tail integration cell intensity.

- **Principle of the Comet Assay**

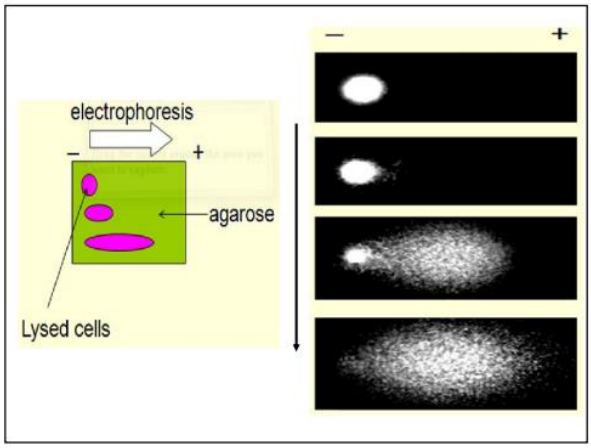
In the Comet Assay process, cell damage is determined by employing the theory that when a charge is applied to a cell, the amount of DNA that leaves the nucleus is an indication of the amount of DNA damage.

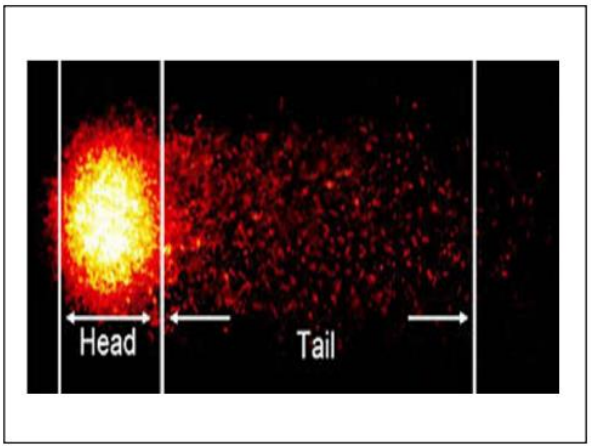
In the process, the single cells under investigation are placed in low melting point agarose which is a gel-like polysaccharide derived from seaweed. This is often carried out on a microscope slide where the cell membrane is broken down (lysed) with the use of a detergent and high salt. This helps the cells to form nucleoids with the tightly coiled DNA in the nuclear matrix.

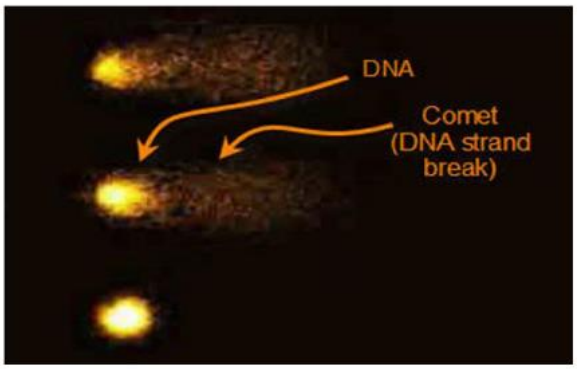
The next stage is electrophoresis - a charge is applied to the cells by using an electrical field. The damaged DNA, which has a negative charge, starts to move out of the nucleus towards the positively charged anode. The undamaged DNA stays where it is.

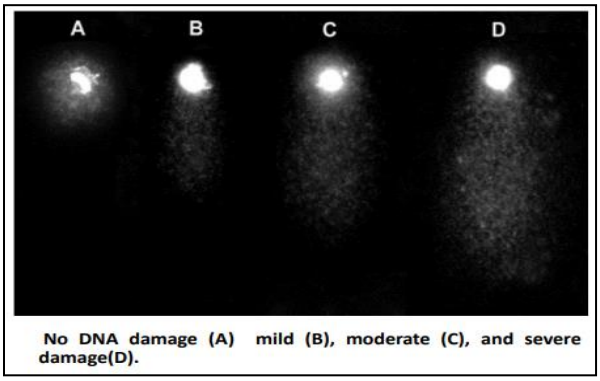
As the damaged DNA travels through the agarose, it creates trails like comet tails – this is visualized with fluorescence microscopy. The intensity, brightness and length of the comet tail relative to the head indicate the number of DNA breaks.

The underlying principle is likely that DNA breakage leads to a loss of supercoiling and results in the freedom to migrate towards the anode.









- **Comet Assay formats**

There are two kinds of Comet Assay. In a Neutral Comet Assay, the DNA is kept as double strands so that the assay is used to detect double-stranded breaks. In contrast, an alkaline Comet Assay is carried out at pH8 and a denaturing step is included so that both single and double-stranded breaks can be determined.

- **Comet assays applications**

I. ***Chronic and degenerative diseases***

DNA damage is a contributing factor for many diseases – the alkaline comet assay can determine the extent of this. Such damage can be found in long-term diseases such as

diabetes mellitus (in which the body is unable to produce enough insulin) and rheumatoid arthritis (characterized by inflammation and pain in the joints).

Additionally, neurodegenerative diseases such as Alzheimer's disease can cause DNA damage. Oxidative stress may be a likely cause of the DNA damage in these diseases. It can result when free radicals are formed from either essential internal metabolic processes or environmental sources including exposure to radiation (X-rays, ozone) or chemicals (smoking, air pollutants).

II. ***Cancer treatment (chemotherapy and radiotherapy)***

In the absence of appropriate cellular regulation and repair, DNA damage and abnormalities can accumulate and lead to the development and progression of cancer. Scientists have studied the presentation and extent of DNA damage in different forms of cancer as a tool to help with diagnosis. The accumulated information has the potential to help doctors treat patients who are undergoing cancer treatment.

Comet assay techniques can also be used in the screening of compounds for use in cancer medication for patients. The process offers a way to determine the sensitivity of tumors to radiotherapy or chemotherapy treatments. An alkaline comet assay has already been employed in these tests to provide information about the sensitivity of tumors to doses of radiation. It has been found to be valuable in colon, prostate and breast cancers.

Assessing the extent of DNA cross-linking and alkylation in combination with DNA strand breakage can also be useful when determining the response of tumors to chemotherapeutic treatments. In fact, scientists have found that there is a clear dose-response and time-response for DNA double strand breaks which can be obtained via comet assay methods.

III. ***Evaluation of geno-toxicity***

Genotoxic and non-genotoxic chemicals can be differentiated through comet assays. This can be helpful in identifying the genotoxic impact of chemicals and carcinogens. The comet assay can even be used as a biomarker to give some indication of genetic susceptibility in addition to detecting potentially carcinogenic DNA damage and assessing cell and tissue genotoxicity.

IV. ***Biomonitoring of occupational and environmental exposure***

A comet assay is useful to detect the level of exposure of populations to genotoxic chemicals and pollutants present in the environment such as those used in manufacturing processes. The assay can therefore be used to determine the impact of these on the health of people living or working near them. Areas which have been studied include crude oil refineries, factories producing certain chemicals and waste refineries.

The DNA damage occurs in the peripheral blood lymphocyte cells (PBL) due to their exposure to metals, chronic and low doses of radiation, volatile organic compounds and

antineoplastic drugs. The resultant DNA damage shows a correlation with micronucleus assay results and chromosomal defects.

A comet assay is used here due to the higher sensitivity, speed and reproducibility offered in comparison to other assays which could be used to study the DNA damage instead.

Exposure of cells to genotoxic substances: Genotoxic substances should be treated with extreme care. Cells can be exposed while in culture by adding directly to the growth media or while embedded in agarose on the slide during the SCGE assay. Chemicals, preferably dissolved in PBS, can be applied to the slides by adding 50 µl of solution per section (half) of the microscope slide and dispersing with a cover slip, or alternatively slides can be immersed in the chemical solution (e.g. hydrogen peroxide). Incubate on ice to inhibit the endogenous DNA repair enzymes.

- ## Single Cell Gel Electrophoresis Assay Protocol

Pre-coating of Glass Microscope Slides:

1. Dip a glass microscope slide into 1.5% molten agarose (PN 75817) made up in distilled/filtered water. Immediately wipe all agarose off the back of the slide.

2. Place slide on bench top or in drying oven and allow the agarose to dry completely. Slides can be used for up to 1 week when stored at RT.

Embedding of Cells:

3. Centrifuge cells (e.g. cells grown in culture or trypsinized cells from plates) at 2000 x g in a microcentrifuge for 1 min at 4°C, discard the supernatant.

4. Resuspend cells in 1 ml of 1X PBS (10X PBS, PN 75889).

5. Centrifuge cells at 100 x g in a microcentrifuge for 5 min at 4°C, discard the supernatant.

6. Resuspend cells in PBS to about 25000 cells per ml.

7. Mix 5 µl of cell suspension (about 125 cells) with 35 µl 0.8% Agarose, Low Melt (PN 32829) (0.8% LM agarose can be microwaved to liquefy and stored at 37°C until use). Pipette mixture immediately onto half a microscope slide and cover with a 22 x 22 mm cover slip.

8. Repeat application of cells for second half of slide.

9. Place slides on ice or at 4°C for 5 min to allow agarose to form gel.

10. Remove cover slips.

11. Cells are now accessible for additional manipulation (e.g. exposure to UV irradiation or to chemical agents).

Lysis of Cells:

12. Cover slides in staining trays with ice cold Lysis Solution. (Note: Triton X-100 lyses the cells. High salt removes histones from DNA.)

13. Incubate on slow shaker for 1 hr at 4°C. Lysis times may vary depending on the cells being used.

14. Rinse slides with filtered water.

15. Wash slides in filtered water 3 times for 5 min each wash.

Treatment of Slides with DNA Modifying Enzymes (Optional, Mut M example):

16. Dilute Mut M (Fpg) 1 x 102 to 103 in reaction buffer (PN 71430).

17. Add 50 µl of enzyme solution per half of microscope slide. Cover with a cover slip.

18. Incubate at 37°C for 30 min.

19. Remove cover slip.

Denaturation/DNA Unwinding:

20. Cover slides with Denaturation Solution.

21. Incubate with slow shaking for 20 min at 4°C.

Electrophoresis:

22. Pre-equilibrate slides by covering with 1X TBE (5X TBE, PN 75891) buffer for 10 min. Place in horizontal submarine gel electrophoresis unit.

23. Electrophorese samples in 1X TBE. For a 13 cm electrode distance, electrophorese for 3-1/2 min at 25V, 3mA. Vary time of electrophoresis as appropriate.

24. Wash slides in filtered water 2 times for 5 min each wash.

25. Slides can be stained immediately or dried completely for later use. For drying, slides can be dipped in 100% EtOH (or MeOH) and dried at RT or 37°C.

Electrophoresis Alternative: Slides can be electrophoresed in fresh Denaturing Solution at 25 volts for 10 min for ss and ds DNA breaks. Electrophoresis time may vary. Neutralize in 0.4M Tris, pH 7.5 (1M Tris, pH 7.5, PN 22639)

Visualization of DNA: SYBR® Green I Nucleic Acid Stain - Dilute SYBR Green I stock 1:10,000 in TE Buffer (pH 7.5-8.0) as directed by manufacturer. Pipet 100 µl diluted SYBR Green I onto dry slides and add cover slips.

Visualize under fluorescence microscope.

Ethidium Bromide - Pipette 80 µl (2 drops) of 2 µg/ml EtBr (PN 75816) on to dry slide and apply cover slip. Incubate for 30 min at room temperature. Remove cover slips and dip slides in filtered H_2O to rinse. Visualize under fluorescence microscope.

Silver Stain - Silver stained slides have the advantage of visualization on a light microscope and slides can be stored for future reference. For detailed protocol, see reference 10.

- **Troubleshooting:**

No comets on Control or Experimental slides.

1. Cells not lysed.

- Extend incubation time in lysis buffer.

2. Lysis buffer not effective.

- Test alternative lysis buffers.

3. DNA not electrophoresed.

- Increase voltage.

- Increase electrophoresis time.

4. DNA not fully denatured.

- Increase denaturing time.

All cells, Control and Experimental, give comets.

1. DNA damage.

- Use chilled buffers; substitute Hank's Balanced Salt Solution (HBSS) for PBS

- Shorten time between cell harvest and start of assay.

- Reduce cell exposure to fluorescent light in work area.

- 0.8% LM agarose was too hot, cool to 37°C.

2. Electrophoretic field too high.

- Reduce electrophoretic time.

- Reduce voltage.

Agarose lifting off of slide.

1. Too much agitation during washes/incubation steps.

- Reduce agitation rate of shaker.

- Perform washes and incubations on the bench top without agitation.

2. Hydrated for too long.

- Dry slides completely before staining DNA (after electrophoresis).

Comets on a slide vary widely in tail length and orientation of tail.

1. Current through the gel was inconsistent because the gel layer was not uniform.

- Use cover slips when dispensing the agarose + cells onto the slide to create a uniform gel layer.

Comets near edges of gel layer do not match comets on rest of slide.

An 'edge effect' in the assay. Do not score comets along edge of gel layer.

Probable questions:

1. Define Comet assay?
2. What is Comet?
3. Describe the protocol of Comet assay?
4. Name the buffers which are used in Comet assay.
5. Discuss the application of Comet assay.

Suggested readings:

1. Scorecomets.com on Comet Assay: http://www.scorecomets.com/about-the-comet-assay/what-is-the-comet-assay
2. Scorecoments.com on how a Comet Assay works: http://www.scorecomets.com/about-the-comet-assay/how-does-it-work
3. US National Library of Medicine, National Institutes of Health: http://www.ncbi.nlm.nih.gov/pubmed/15004294
4. AMSBIO on Comet Assay
5. AMSBIO presentation on Comet Assay

UNIT XI

FRAP assay, FRET assay

Objective: In this unit we will discuss about FRAP assay, FRET assay.

FRAP assay

The ferric reducing antioxidant power (FRAP) assay is a typical ET-based method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in an acidic medium.

Ferric reducing antioxidant power (FRAP) assay is a widely used method that uses antioxidants as reductants in a redox-linked colorimetric reaction, wherein Fe^{3+} is reduced to Fe^{2+}. Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion reduction at low pH causes formation of a colored ferrous-probe complex from a colorless ferricprobe complex. Antioxidants are molecules which act as reducing agents by donating electrons to free radicals to stabilize them and minimize the damage caused by free radicals to DNA, cells, and organ systems. Antioxidants include substances such as polyphenols, flavonoids, vitamins, and enzymes like glutathione peroxidase and superoxide dismutase. They are known to have beneficial health effects such as lowering the risk of cancer, heart disease, and neurodegenerative disorders and are abundantly found in plants, fruits, vegetables, beverages, and natural products. The Ferric Reducing Antioxidant Power (FRAP) Assay Kit provides a quick, sensitive, and easy way for measuring antioxidant capacity of various biological samples. The assay is high-throughput adaptable and can detect antioxidant capacities as low as 0.2 mM Fe^{2+} equivalents.

- ### Procedure of FRAP assay

I. Sample Preparation

A variety of fruit, vegetable, and plant samples, beverages as well as serum and plasma can be used with this assay. Fruit, vegetable and plant extractions can be done using acid-methanol (e.g., a mixture of methanol:ultrapure water:1 M HCl at a ratio of 70:29.5:0.5), acid-ethanol, or acetone extraction methods. Users can use the extraction methods of their choice for their particular samples with proper dilutions to ensure the values fall within the standard curve range. Do not use FRAP Assay Buffer for extraction of samples. Fruit and vegetable juices, herbal products, and freeze-dried fruits solubilized in suitable solvents, beverages such as wines, green tea, and coffee can also

be used directly with appropriate dilutions while making sure potential interfering substances do not give a significant background.

For unknown samples, perform a pilot experiment and test several doses to ensure the readings are within the Standard Curve range. Also ensure that the reaction is complete within 60 minutes for the absorbance readings. For samples exhibiting significant background, prepare parallel sample well(s) as background controls.

Add 10 mL of sample per well. For the positive control, add 4 mL of the FRAP Positive Control plus 6 mL of FRAP Assay Buffer into desired well(s).

II. Standard Curve Preparation

Prepare Ferrous Standards in desired wells of a clear flat-bottom 96 well plate according to Table 1.

Table 1. Preparation of Ferrous Standards

Well	Ferrous Standard (2 mM)	FRAP Assay Buffer	Ferrous (nmol/well)
1	0μL	10μL	2
2	2μL	8μL	4
3	4μL	6μL	8
4	6μL	4μL	12
5	8μL	2μL	16
6	10μL	0μL	20

Reaction Mix Mix enough reagents for the total number of wells to be assayed including standards, samples, positive control, and background control(s). For each well, prepare190 μL of Reaction Mix according to Table 2.

Table 2. Preparation of Reaction Mix

Reagent	Reaction Mix	Background Control Mix
FRAP Assay Buffer	152μL	171μL
$FeCl_3$ Solution	19μL	19μL
FRAP Probe	19μL	-

Mix and add 190μL of the Reaction Mix to each well containing the standards, positive control, and test samples. For background correction, add 190μL of Background Control Mix (without FRAP probe) to sample background control well(s) and mix well.

III. Measurement

Measure absorbance immediately at 594 nm (A594) kinetic mode for 60 minutes at 37°C Use the absorbance values obtained at 60 minutes for further calculations (ensure the reaction is complete at 60 minutes). If desired, the Ferrous Standard Curve can be read in endpoint mode (i.e., at the end of the 60 minute incubation time).

IV. Results

1. Subtract the 0 nmol Standard reading from all Standard Curve readings.

2. Plot the Ferrous Standard Curve.

3. If the sample background control reading is significant, subtract the background control reading from its paired sample reading.

4. Compare the sample A594 values to the Ferrous Standard Curve to get nmol of reduced Ferrous ions generated during the reaction (B).

5. Use the following calculation to determine mM Ferrous equivalents of the samples.

Sample FRAP or mM Ferrous Equivalent (nmol/ mM Fe^{2+} equivalents) = $\frac{B \times D}{V}$

where:

B = Ferrous ammonium sulphate amount from Standard Curve (nmol)

D = Sample dilution factor

V = Sample volume added into the reaction well (μL)

- **What is the FRAP assay used for?**

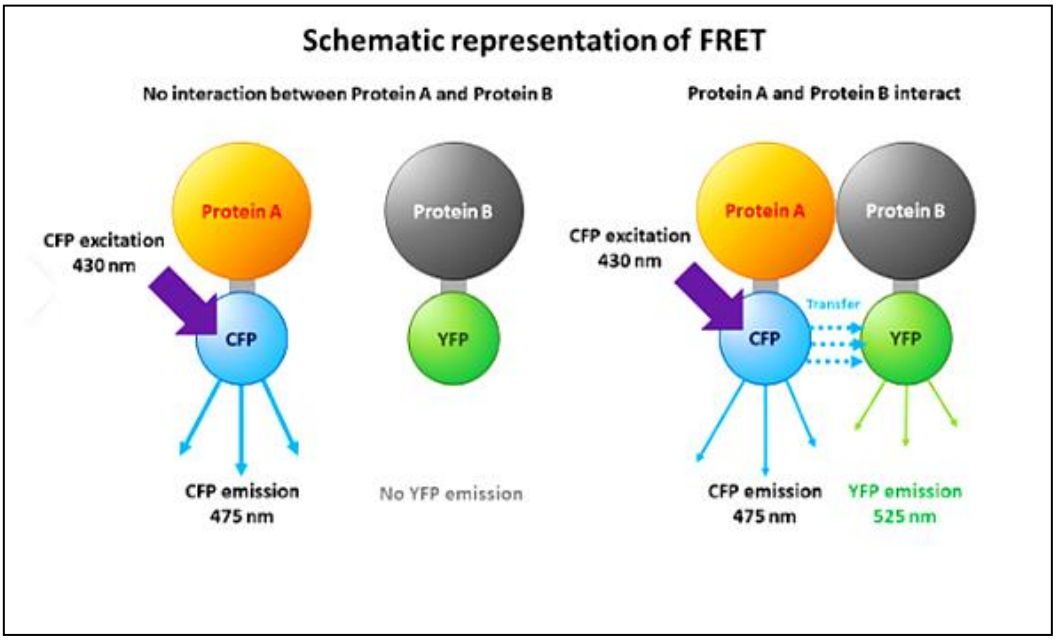
The FRAP assay is used to measure the antioxidant power based on the reduction at low pH of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to an intense blue color ferrous-tripyridyltriazine complex (Fe^{2+}-TPTZ) with an absorption maximum at 593 nm.

FRET assay

Fluorescence resonance energy transfer (FRET)* is a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-range dipole–dipole coupling.

- **FRET principle**

FRET is based on the fact that a donor dye (e.g. CFP) in an excited state can transfer a part of its energy through nonradiative dipole–dipole coupling to an acceptor molecule like YFP. The technology involves fusion of donor and acceptor fluorescent proteins to molecules of interest. Co-expression of fusion constructs in living cells enables their interaction to be studied in real time in a quantitative manner. The emission from the acceptor can be detected as soon as both dyes are in close proximity, e.g. when interaction of two proteins has taken place.



- **What are the advantages of FRET assay?**

FRET has two distinct advantages over typical immunofluoresence techniques. First, the distance required for energy transfer is so short that it allows the probing of molecular interactions, and second, the observable excitation range of the acceptor is very narrow and thus easily observed.

- **Applications of FRET technology**

FRET is an extremely powerful method of identifying potential molecular interactions and can be used in techniques such as flow cytometry, immunocytochemistry, immunohistochemistry and ELISA. FRET is also ideally suited to High Throughput Screening (HTS) since it is simple, sensitive and easily automated.

One popular use of FRET is to identify an interaction between two biomolecules, for example, the binding of a ligand to a receptor; a FRET signal can only be detected when the biomolecules are in close proximity by virtue of a binding event.

FRET relies on the use of high quality labeled reagents. Depending on the intended assay setup these could be antibodies, proteins or peptides.

- **What are the limitations of FRET assay?**

The limitation of FRET is that this transfer process is effective only when the separating distance of donor-acceptor pair is smaller than 10 nanometers. However, FRET is a highly distance-dependent phenomenon and thus has become a popular tool to measure the dynamic activities of biological molecules within nanoscale.

Probable questions:

1. Write down the full form of FRAP and FRET?
2. What is the application of FRAP assay?
3. What is the procedure of FRAP assay?
4. What are the limitations of FRET assay?
5. Write down the principle of FRET assay with diagram.

Suggested readings:

1. Nature Protocols on The comet assay: a method to measure DNA damage in individual
   cells: http://www.nature.com/nprot/journal/v1/n1/full/nprot.2006.5.html
2. Journal of Clinical & Diagnostic Research on comet assay applications: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4413081/
3. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3249911/
4. https://www.creative-biostructure.com/fluorescence-resonance-energy-transfer-fret-assay-306.htm

UNIT XII

Immunological assay: Monoclonal and polyclonal antibody generation

Objective: In this unit we will discuss about Immunological assay: Monoclonal and polyclonal antibody generation.

Introduction

Immunological assays are a biochemical test that measures the presence or concentration of a macromolecule or a small molecule in a solution through the use of an antibody or an antigen. Immunological assays come in many different formats and variations, and may be run in multiple steps with reagents being added and washed away or separated at different points in the assay. Enzymes are popularly used in these immunological assays to track antibodies and antigens.

The principle behind the Immunoassay test is the use of an antibody that will specifically bind to the antigen of interest.

The antibodies used in the Immunoassay must have a high affinity for the antigen. The antibodies used in the Immunoassay can either be monoclonal or polyclonal antibodies.

The differences between the two antibodies are:

· Monoclonal antibodies only bind to very specific antigens and therefore give more accurate and specific results, but these antibodies tend to be more expensive.

· Polyclonal antibodies are inexpensive but can recognise multiple epitopes on an antigen or they can recognize multiple antigens and these antibodies tend to be less specific.

- ## Why we need immunoassays

Immunoassays can detect antigens of interest in very low concentrations that cannot be measured by standard tests. Immunoassays are generally quick and specific which enables a clinician to quickly and accurately diagnose a range of diseases such as diabetes, cancer and heart disease.

- ## Monoclonal antibody production

What is a Monoclonal Antibody?

A Monoclonal antibody, by contrast, represents antibody from a single antibody producing B cell and therefore only binds with one unique epitope. Each individual antibody in a polyclonal mixture is technically a monoclonal antibody; however, this

term generally refers to a process by which the actual B-cell is isolated and fused to an immortal hybridoma cell line so that large quantities of identical antibody can be generated.

➢ Hybridoma technology for production of monoclonal antibodies:

- Monoclonal antibodies are produced by hybridoma technology.
- The term hybridoma is used to fused cells resulting due to fusion of following two types of cells-a lymphocytes and tumor cell.
  - An antibody producing B- lymphocytes (eg. Spleen cell of mouse immunized with RBCs from sheep)
  - A single myeloma cell (eg. Bone marrow tumor cell) that can adopted to grow for infinite time in culture
- The fused product derived the ability of two different types of cells. ie. Ability to produce large amount of pure antibodies as lymphocytes and ability to grow or multiply indefinitely like tumor cell.

➢ Steps in production of monoclonal antibodies:

Step I: Immunization of rabbit or rat and extraction of B-lymphocytes

- In order to isolate B-lymphocyte producing certain antibodies, rabbit or lab rat is immunized through repeated injection of specific antigen (sheep RBCs)
- A sample of B-cells is extracted from spleen of rabbit or rat

Step II: fusion of myeloma cell with B-lymphocytes:

- The extracted B-lymphocytes is added to a culture of myeloma cell from bone marrow.
- The intended result is the formation of hybridoma cells formed by fusion of B-cell and myeloma cell.
- The fusion is done by using Polyethylene glycol (PEG) or by electrophoration or by using phages.

Step III: selection of hybridoma cell

- The next step is selection of hybridoma cells.
- The B-lymphocytes contains HPRT1 gene which codes for enzyme Hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The enzyme HGPRT involved in synthesis of nucleotides from Hypoxanthine present in culture medium. Therefore B- cells can grow in medium containing Hypoxanthine amonopterin thymine (HAT media).

- But myeloma cell lack HPRT1 gene so, it does not produce HGPTR enzyme and it does not grow in HAT medium.
- The myeloma cell fused with another myeloma cell or those do not fused at all die in HAT medium since they do not utilize Hypoxanthine.
- Similarly, B- cell that fuse with another B- cell or those do not fuse at all die eventually because they do not have capacity to divide indefinitely,
- So, only hybridoma cell ie. fused cell between myeloma and B-cell can survive and divide in HAT medium.
- Screening is done to select hybridoma cells which are the desired cell for monoclonal antibodies production.

Step IV: culture of Hybridoma cell:

- The selected hybridoma cells are cultured in suitable culture medium, often supplemented with insulin, transferon, ethanol, amine and other additional hormones.
- Some commonly used culture media for hybridoma cell for production of monoclonal antibodies are:
  - DMEM (Dulbecco's modified eagle medium)
  - IMDM (Iscove's Modified Dulbecco's Medium)
  - Ham's F12
  - RPMI 1640 medium (Roswell Park Memorial Institute 1640 medium)

Step V: Inoculation of hybridoma cell into suitable host

- These hybridoma cells are then injected into lab animal so that they starts to produce monoclonal antibodies.
- These hybridoma cells may be frozen and store for future use.

Step VI: extraction and purification of Monoclonal antibodies:

- Monoclonal antibodies from host animal is extracted and purified by one of the following methods;
- Ion exchange chromatography
- Antigen affinity chromatography
- Radial immunoassay
- Immune precipitation

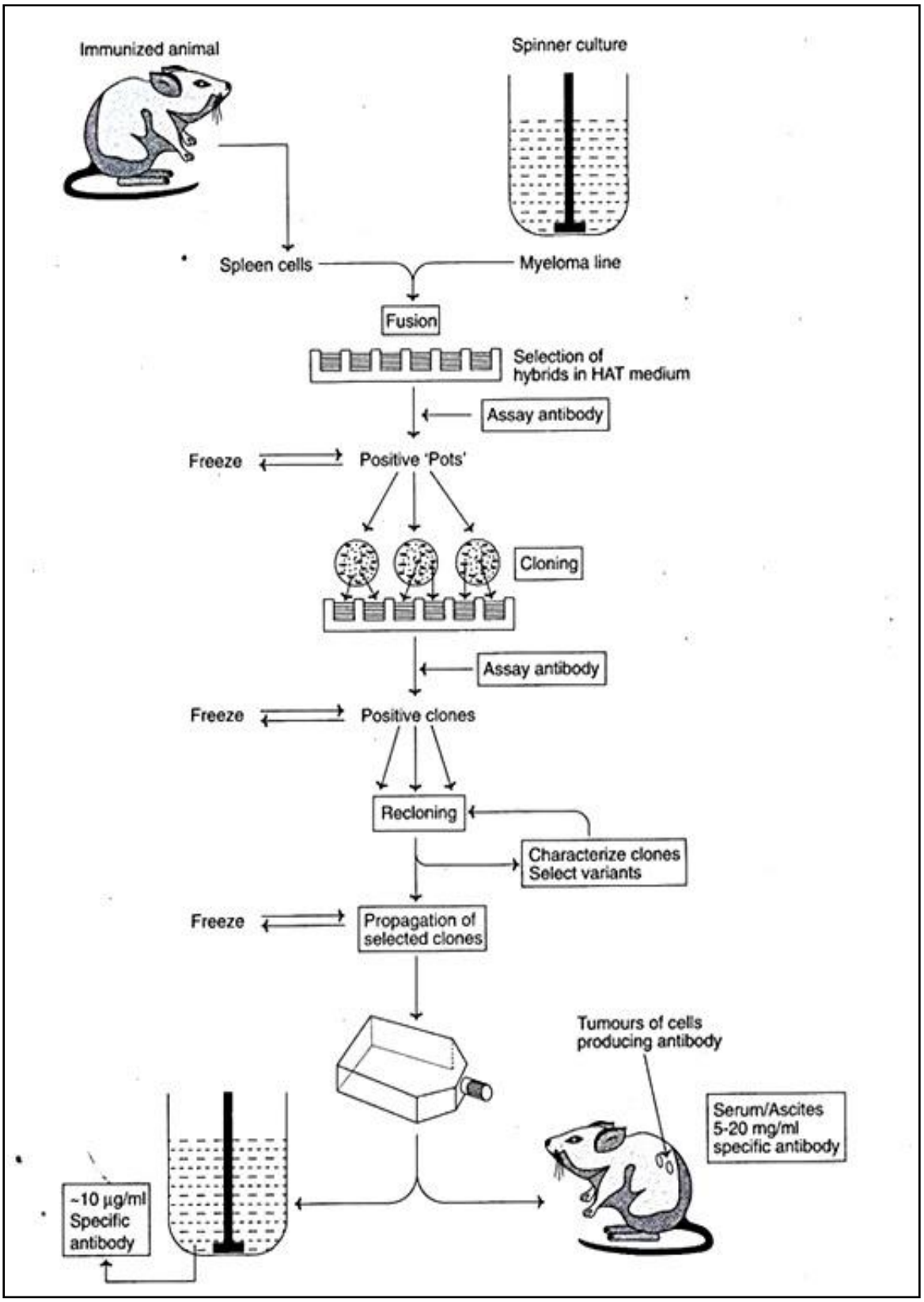


Fig 2: Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas

Advantages of monoclonal antibody:

- Can produce large quantities of identical antibody. Batch to batch homogeneity.
- High specificity to a single epitope. Reduced probability of cross reactivity.
- Can provide better results in assays requiring quantification of the protein levels.

Disadvantages of monoclonal antibody:

- Significantly more expensive to produce.
- Requires significantly more time to produce and develop the hybridized clone.
- Small changes in the epitope's structure often render the monoclonal antibody unable to detect the target protein.
- More demanding storage conditions for the clone.
- Cell culture and purification capabilities required.
- Less robust for detecting the protein in a denatured state or altered conformation.
- Less ideal for application requiring quick capture of the target protein.
- More sensitive to pH and buffer conditions.
- More susceptible to binding changes when labeled.
- To offset many of these disadvantages, it is necessary to produce a pool of several monoclonal antibodies. This is typically cost and time prohibitive.

Polyclonal Antibodies

Polyclonal antibodies (pAbs) are a complex mixture of several antibodies that are usually produced by different B-cell clones of an animal. These antibodies recognize and bind to many different epitopes of a single antigen and hence can form lattices with the antigens.

➢ How to Make Polyclonal Antibodies

What is polyclonal antibody production? Polyclonal antibodies (pAbs) are produced by injecting a specific antigen into lab animals, such as rabbits and goats, etc. The animal is immunized repeatedly to obtain higher titers of antibodies specific for the antigen. Within a few weeks, these polyclonal antibodies can be harvested and collected from the antiserum.

Production of polyclonal antibodies is easier and more cost-effective than the production of monoclonal antibodies. Furthermore, polyclonal antisera can be

generated in a shorter time (4-8 weeks), whereas it takes about 3 to 6 months to produce mAbs.

Sino Biological provides polyclonal antibody production services which deliver you antisera or purified antibodies in just 45 days. Just send us an antigen sequence, and we'll get you high-affinity polyclonal antibodies for your research or diagnostic purposes.

Production of Fab Antibodies

There are several critical steps involved in the production of polyclonal antibodies in rabbits. A four-step procedure of pAb production is outlined below.

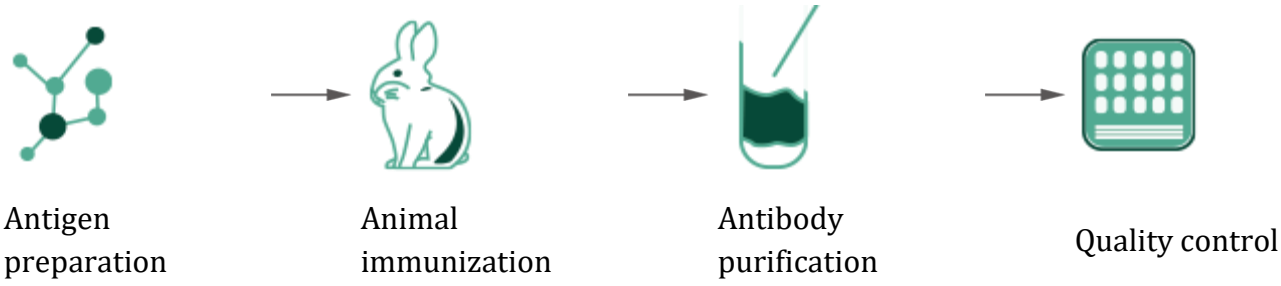


Fig 1. How to generate polyclonal antibodies

Step 1: Antigen preparation

The production process starts with preparing the protein or peptide antigens. It's important to ensure the quality of the target antigen, in particular when binding to a conformational epitope is desired. The specificity of polyclonal antibody obtained depends on the purity of the antigen applied. Impurities (<1%) may prove to be immunodominant (e.g., with many bacterial antigens) and may result in antibodies that have more activity against the impurity than against the antigen of interest. Purification of antigen is a time-consuming and laborious work.

Luckily, Sino Biological offers antigen preparation service which is based on our recombinant protein expression platforms. We have rich experience of recombinant protein expressed in CHO, HEK293 and *E. coli*.

Step 2: Animal immunization

The choice of animals for the production of polyclonal antibodies depends upon the amount of antiserum desired, the evolutionary distance between the species from which the protein of interest has been derived and the species of the animal to be immunized, and prior experience with the immunogens. Rabbits are the usual animal of choice because they are genetically divergent from the human and mouse sources of the

proteins most often studied. Rabbits provide as much as 25 ml of serum from each bleed without significant harmful effects.

Adjuvants are used to enhance the immune response for pAb production. Freund's Adjuvant is one of the most commonly used adjuvants in research. In the presence of adjuvant, the protein antigen is injected intramuscularly, intradermally, or subcutaneously into an animal of the chosen species. Booster immunizations are started 4 to 8 weeks after the priming immunization and continued at 2- to 3-week intervals. Prior to the priming immunization, following the primary and each booster immunization, the animal is bled and serum prepared from whole blood. When the antibody titer has reached an acceptable level, the production of polyclonal antibodies should be ended.

Step 3: Antibody purification

Affinity purification is a good choice for purification of polyclonal antibodies.

Protein A/G affinity purification can enrich Immunoglobulin G (IgG) in the raw antiserum and remove the bulk of unwanted proteins. However, there's still a large quantity of non-specific IgGs in these preparations. This will considerably increase the background noise when the antibody is used in assays such as ELISA, immunohistochemistry, and Western blot, etc.

To isolate specific polyclonal antibodies from antiserum, antigen-specific affinity purification is often used. Antigen affinity purification results in the elimination of the bulk of the non-specific IgG fraction, and enriches the fraction of immunoglobulin that specifically reacts with the target antigen.

Step 4: Quality control

After purification, a series of quality control tests are performed to ensure the quality of polyclonal antibodies. Antibody concentration is evaluated by absorption at 280 nm (A280). The purity of polyclonal antibody is checked using SDS-PAGE. To estimate the polyclonal antibody titer, an ELISA test was the most suitable method.

Finally, labeling polyclonal antibody with HRP or biotin provides sensitive enzymatic detection in IHC and the flexibility of using a variety of conjugated avidin, respectively.

Advantages of polyclonal antibody:

- Inexpensive to produce.
- Quick to produce. Purified antibody ready to use in under four months.
- Easy to store.
- Highly stable and tolerant of pH or buffer changes.

- Higher overall antibody affinity against the antigen due to recognition of multiple epitopes.
- In general, ability to detect multiple epitopes gives more robust detection.
- Offers greater sensitivity for detecting proteins that are present in low quantities in a sample since multiple antibodies will bind to multiple epitopes on the protein.
- Ideal as the capture antibody in a Sandwich ELISA. Greater ability to quickly capture the target protein.
- Superior antibody affinity generally results in quicker binding to target antigen. Ideal in assays requiring quick capture of the protein such as IP or ChIP.
- Significantly more robust when assaying proteins that show slight variations in individual epitopes such as denaturation, polymorphism or conformational changes.
- Superior for use in detecting a native protein in multiple assay types.
- Much easier to couple with antibody labels. Less likely to affect binding capability.

Disadvantages of polyclonal antibody:

- Variability between different batches produced in different animals at different times
- Higher potential for cross reactivity due to recognizing multiple epitopes
- Affinity purification of the serum will typically be required to minimize cross reactivity

How are polyclonal antibodies used?

pAbs have wide range of applications, including diagnostic testing as well as qualitative and quantitative biological analyses. For example, pAbs are used in immunofluorescence and immunohistochemical techniques such as sandwich ELISA to detect tumor markers and other proteins of interest.

pAbs are also used for mediation or modulation purposes such as in immunotherapy, active signaling, or for neutralizing activities. An example of this is the use of pAbs in the treatment of digoxin Immune Fab in fatal digoxin toxicity.

pAbs such as Rho (D) immune globulin is injected into mothers with Rhesus-negative blood group to prevent hemolytic disease in a newborn. Rho (D) is produced from a pool of human plasma collected from Rhesus-negative donors who have antibodies for the D antigen (present on red blood cells).

pAbs also find applications in histopathological analyses that employ immunoperoxide staining. Apart from these applications, pAbs are used in immunoaffinity purification for purification or enrichment of antigens.

Recombinant pAbs are used in cancer therapy due to their ability to multi-target tumor cells compared to monoclonal antibodies. Although monoclonal antibodies are widely used in cancer therapy, relapse is common due to the emergence of tumor cells that are resistant to the antibody. By using pAbs, diverse recombinant antibodies can be developed that cross-react with different types of cancers.

The way forward shows recombinant pAbs being used to minimize unnecessary polyreactivity as seen when traditional pAbs are used. Use of pAbs in different assays not only generates high throughput but may also be used to develop specific antibodies for human gene products that are also renewable.

Polyclonal vs. monoclonal antibodies

This summary table highlights the five main differences between the two types of antibodies.

Polyclonal antibodies	**Monoclonal antibodies**
Refer to a mixture of immunoglobulin molecules that are secreted against a particular antigen.	Refer to a homogenous population of antibodies that are produced by a single clone of plasma B cells.
Produced by different clones of plasma B cells.	Produced by the same clone of plasma B cells.
Production does not require hybridoma cell lines.	Production requires hybridoma cell lines.
A heterogeneous antibody population.	A homogenous antibody population.
Interact with different epitopes on the same antigen.	Interact with a particular epitope on the antigen.

Probable questions:

1. What is monoclonal antibody?
2. What is hybridoma?
3. Describe the detail procedure of monoclonal antibody generation.
4. What are the advantages and disadvantages of monoclonal antibody application?
5. What is polyclonal antibody?
6. Describe the detail procedure of polyclonal antibody generation.
7. What are the advantages and disadvantages of polyclonal antibody application?
8. Write down the differences between monoclonal antibody and polyclonal antibody.

Suggested readings:

1. Snustad D P, Simmons MJ. 2009. Principles of Genetics. V Edition. John Wiley and Sons Inc
2. Strickberger M. W – Genetics; Macmillam
3. Tamarin R. H. – Principles of Genetics; McGraw Hill
4. Klug W S, Cummings MR, Spencer CA. 2012. Concepts of Genetics. Xth Ed. Benjamin Cummings
5. Leenaars, P. M., Hendriksen, C. F., de Leeuw, W. A., Carat, F., Delahaut, P., Fischer, R., ... & Lindblad, E. B. (1999). The Production of Polyclonal Antibodies in Laboratory Animals: The report and recommendations of ECVAM workshop 35. Alternatives to laboratory animals, 27(1), 79-102.
6. Leenaars, M., & Hendriksen, C. F. (2005). Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. Ilar Journal, 46(3), 269-279.
7. http://asheducationbook.hematologylibrary.org/content/2000/1/394.full
8. https://academic.oup.com/ilarjournal/article/46/3/258/738903#36185770
9. https://www.tandfonline.com/doi/abs/10.4161/19420862.2015.989047
10. https://onlinelibrary.wiley.com/doi/full/10.1002/jcb.20536

UNIT XIII

Hybridoma technology

Objective: In this unit we will discuss about Hybridoma technology.

Introduction

Antibodies or immunoglobulin's are protein molecules produced by a specialized group of cells called B-lymphocytes (plasma cells) in mammals. The structures, characteristics and various other aspects of immunoglobulin's (Igs) are described elsewhere. Antibodies are a part of the defense system to protect the body against the invading foreign substances namely antigens.

Each antigen has specific antigen determinants (epitopes) located on it. The antibodies have complementary determining regions (CDRs) which are mainly responsible for the antibody specificity. In response to an antigen (with several different epitopes), B-lymphocytes gear up and produce many different antibodies. These types of antibodies which can react with the same antigen are designated as polyclonal antibodies.

The polyclonal antibody production is variable and is dependent on factors such as epitopes, response to immunity etc. Due to lack of specificity and heterogenic nature, there are several limitations on the utility of polyclonal antibodies for therapeutic and diagnostic purposes.

Monoclonal antibody (MAb) is a single type of antibody that is directed against a specific antigenic determinant (epitope). It was a dream of scientists to produce MAbs for different antigens. In the early years, animals were immunized against a specific antigen, B-lymphocytes were isolated and cultured in vitro for producing MAbs. This approach was not successful since culturing normal B-lymphocytes is difficult, and the synthesis of MAb was short-lived and very limited.

It is interesting that immortal monoclonal antibody producing cells do exist in nature. They are found in the patients suffering from a disease called multiple myeloma (a cancer of B- lymphocytes). It was in 1975. George Kohler and Cesar Milstein (Nobel Prize, 1984) achieved large scale production of MAbs. They could successfully hybridize antibody—producing B-lymphocytes with myeloma cells in vitro and create a hybridoma.

The result is that the artificially immortalized B-lymphocytes can multiply indefinitely in vitro and produce MAbs. The hybridoma cells possess the growth and multiplying properties of myeloma cells but secrete antibody of B-lymphocytes. The production of monoclonal antibodies by the hybrid cells is referred to as hybridoma technology.

Monoclonal antibodies (Mabs)

- Antibodies are glycoprotein synthesized in blood against specific antigens hust to combat and give immunity. Such antibodies are heterogenous and are polyclonal antibodies. Therefore they do not have characteristics of specificity.
- If a specific lymphocyte after isolation and culture invitro becomes capable of producing a single type of antibody which bears specificity against specific antigen, it is known as monoclonal antibody.
- These monoclonal antibodies are derived from a single clone of cell which recognize only one kind of antigen.
- Monoclonal antibodies are produced against a variety of proteins, glycoproteins, glycolipids, nucleic acids and chemically defined groups linked to protein carriers.

Principle for Creation of Hybridoma Cells:

The myeloma cells used in hybridoma technology must not be capable of synthesizing their own antibodies. The selection of hybridoma cells is based on inhibiting the nucleotide (consequently the DNA) synthesizing machinery. The mammalian cells can synthesize nucleotides by two pathways—de novo synthesis and salvage pathway (Fig.1).

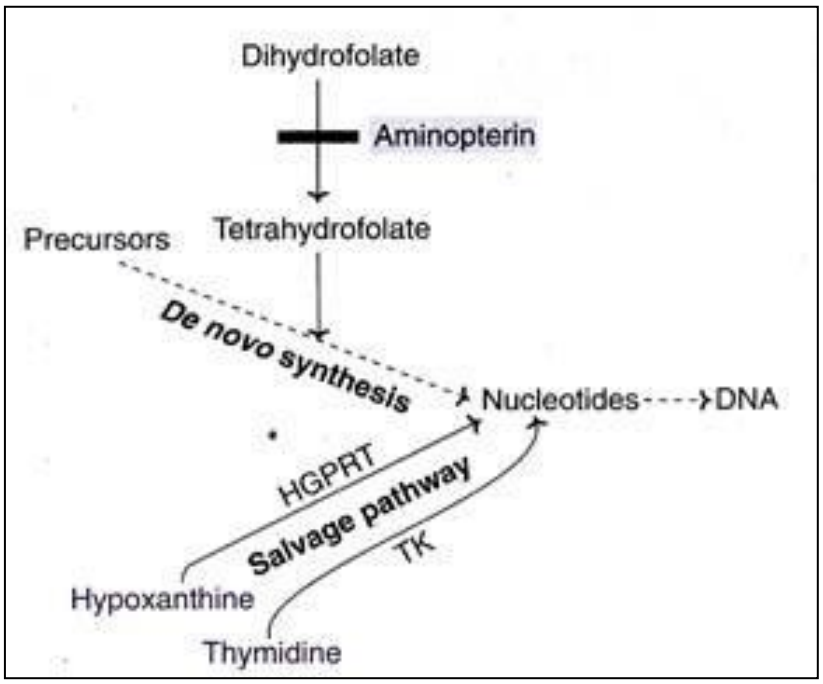


Fig 1: Pathway for the synthesis of nucleotides (HGPRT –Hypoxanthine Guanine Phosphoribosyl Transferase; TK –Thymidine Kinase)

The de novo synthesis of nucleotides requires tetrahydrofolate which is formed from dihydrofolate. The formation of tetrahydrofolate (and therefore nucleotides) can be blocked by the inhibitor aminopterin. The salvage pathway involves the direct conversion of purines and pyrimidine's into the corresponding nucleotides. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a key enzyme in the salvage pathway of purines.

It converts hypoxanthine and guanine respectively to inosine monophosphate and guanosine monophosphate. Thymidine kinase (TK), involved in the salvage pathway of pyrimidine's converts thymidine to thymidine monophosphate (TMP). Any mutation in either one of the enzymes (HGPRT or TK) blocks the salvage pathway.

When cells deficient (mutated cells) in HGPRT are grown in a medium containing hypoxanthine aminopterin and Thymidine (HAT medium), they cannot survive due to inhibition of de novo synthesis of purine nucleotides (Note : Salvage pathway is not operative due to lack of HGPRT). Thus, cells lacking HGPRT, grown in HAT medium die.

The hybridoma cells possess the ability of myeloma cells to grow in vitro with a functional HGPRT gene obtained from lymphocytes (with which myeloma cells are fused). Thus, only the hybridoma cells can proliferate in HAT medium, and this procedure is successfully used for their selection.

Hybridoma technology for production of monoclonal antibodies:

- Monoclonal antibodies are produced by hybridoma technology.
- The term hybridoma is used to fused cells resulting due to fusion of following two types of cells-a lymphocytes and tumor cell.
    - An antibody producing B- lymphocytes (eg. Spleen cell of mouse immunized with RBCs from sheep)
    - A single myeloma cell (eg. Bone marrow tumor cell) that can adopted to grow for infinite time in culture
- The fused product derived the ability of two different types of cells. ie. Ability to produce large amount of pure antibodies as lymphocytes and ability to grow or multiply indefinitely like tumor cell.

Steps in production of monoclonal antibodies:

Step I: Immunization of rabbit or rat and extraction of B-lymphocytes

- In order to isolate B-lymphocyte producing certain antibodies, rabbit or lab rat is immunized through repeated injection of specific antigen (sheep RBCs)
- A sample of B-cells is extracted from spleen of rabbit or rat

Step II: fusion of myeloma cell with B-lymphocytes:

- The extracted B-lymphocytes is added to a culture of myeloma cell from bone marrow.
- The intended result is the formation of hybridoma cells formed by fusion of B-cell and myeloma cell.
- The fusion is done by using Polyethylene glycol (PEG) or by electrophoration or by using phages.

Step III: selection of hybridoma cell

- The next step is selection of hybridoma cells.
- The B-lymphocytes contains HPRT1 gene which codes for enzyme Hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The enzyme HGPRT involved in synthesis of nucleotides from Hypoxanthine present in culture medium. Therefore B- cells can grow in medium containing Hypoxanthine amonopterin thymine (HAT media).
- But myeloma cell lack HPRT1 gene so, it does not produce HGPTR enzyme and it does not grow in HAT medium.
- The myeloma cell fused with another myeloma cell or those do not fused at all die in HAT medium since they do not utilize Hypoxanthine.
- Similarly, B- cell that fuse with another B- cell or those do not fuse at all die eventually because they do not have capacity to divide indefinitely,
- So, only hybridoma cell ie. fused cell between myeloma and B-cell can survive and divide in HAT medium.
- Screening is done to select hybridoma cells which are the desired cell for monoclonal antibodies production.

Step IV: culture of Hybridoma cell:

- The selected hybridoma cells are cultured in suitable culture medium, often supplemented with insulin, transferon, ethanol, amine and other additional hormones.
- Some commonly used culture media for hybridoma cell for production of monoclonal antibodies are:
    - DMEM (Dulbecco's modified eagle medium)
    - IMDM (Iscove's Modified Dulbecco's Medium)
    - Ham's F12
    - RPMI 1640 medium (Roswell Park Memorial Institute 1640 medium)

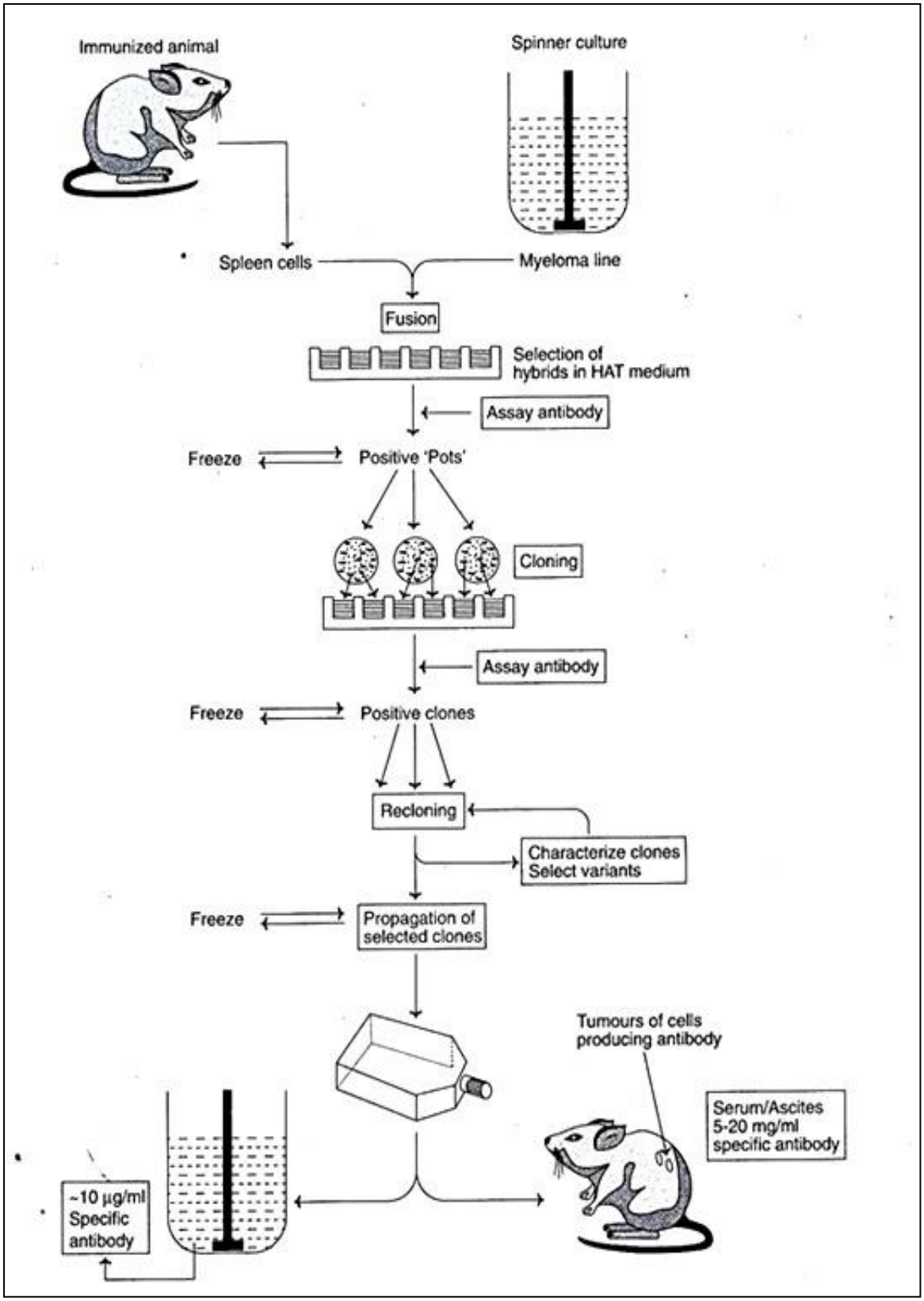


Fig 2: Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas

Step V: Inoculation of hybridoma cell into suitable host

- These hybridoma cells are then injected into lab animal so that they starts to produce monoclonal antibodies.
- These hybridoma cells may be frozen and store for future use.

Step VI: extraction and purification of Monoclonal antibodies:

- Monoclonal antibodies from host animal is extracted and purified by one of the following methods;
- Ion exchange chromatography
- Antigen affinity chromatography
- Radial immunoassay
- Immune precipitation

Application of Monoclonal antibodies:

1. **Disease diagnosis:**

- ELISA to test HIV, hepatitis, Herpes etc
- RIA- to test viral infection
- Mabs to Hunam chorionic gonadotropin

2. Disease treatment

- OKT3- it is an antibody to T3 antigen of T cell which can be used to prevent acute renal allograph rejection in human.
- Different types of Mabs are used in radial immunodetection and radial immune therapy of cancer.

3. Passive immunization or disease prevention

- Monoclonal antibodies based drugs can be used to treat septic shock
- Used as vaccine

4. **Detection and purification of biomolecules**

- Mabs are very useful in determining the presence and absence of specific proteins through western blotting technique.
- Besides that, it can be used to classify strains of a single pathogen. Eg. *Neisseria gonorrhea* can be typed using Monoclonal antibodies.

Probable questions:

1. Define Hybridoma technology?
2. Describe the principle for creation of Hybridoma Cells.
3. What is salvage pathway?
4. What is de novo pathway?
5. What is Monoclonal antibody?
6. Elastrate the steps in production of monoclonal antibodies with diagram.
7. Write down the application of Monoclonal antibody production.

Suggested readings:

1. Snustad D P, Simmons MJ. 2009. Principles of Genetics. V Edition. John Wiley and Sons Inc
2. Strickberger M. W – Genetics; Macmillam
3. Tamarin R. H. – Principles of Genetics; McGraw Hill
4. Klug W S, Cummings MR, Spencer CA. 2012. Concepts of Genetics. Xth Ed. Benjamin Cummings

UNIT XIV

In vitro mutagenesis and gene knockout

Objective: In this unit we will discuss about *In vitro* mutagenesis and gene knockout.

Introduction

Site-directed mutagenesis is the production of either random or specific mutations in a piece of cloned DNA. Typically, the DNA will then be reintroduced into a cell or an organism to assess the results of the mutagenesis.

It is a technique conceived in the 70s used to mutate specific DNA sequences *in vitro*. It relies on synthetic short single-stranded DNA fragments, or *oligonucleotides*, that contain designated mutations to act as templates in the presence of *DNA polymerase* enzyme.

In the late 80s, *Polymerase Chain Reaction (PCR)*, another laboratory technique, was incorporated into site-directed mutagenesis workflow. The modified site-directed mutagenesis is a simple and efficient technique that has rapidly become the cornerstone of gene and protein function studies.

As a result, Michael Smith, the inventor of site-directed mutagenesis, and Kary B. Mullis, the inventor of PCR, were awarded the equally shared 1993 Nobel Prize in Chemistry.

Background and Conception

Originally termed oligonucleotide-directed mutagenesis, the technique was primarily developed in a single-stranded DNA *Escherichia coli* phage ΦX174. The core idea of the technique stemmed from the following discoveries in the bacteriophage:

1. Short oligonucleotides could form a stable double-stranded DNA complex despite a few mismatches in the nucleotides;
2. A small number of bacteriophages, which possess point mutations, could be reverted into wild-type when a wild-type DNA was used as a complementary strand during DNA annealing before transfection;
3. Short oligonucleotides could act as a primer on a circular single-stranded DNA template for DNA replication by *DNA polymerase I* in *E. coli*; and
4. The resulting double-stranded, open circular DNA produced in (3) can be converted into closed double-stranded circular DNA using enzymatic *ligation*.

These discoveries inspired Smith to develop an oligonucleotide-based mutagenic method. In this **method**, a short strand of synthetic oligonucleotides contains the predefined mutation and acts as a mutagen that alters a specific nucleobase on the specified DNA position. The first site-directed mutagenesis reported by Smith and his

team used *one* strand of oligonucleotides as mutagens to prime with a single-stranded circular DNA of the phage ΦX174. Their nucleotide sequences were complementary to the template, except for one nucleobase designated to introduce a nonsense mutation (Figure 1).

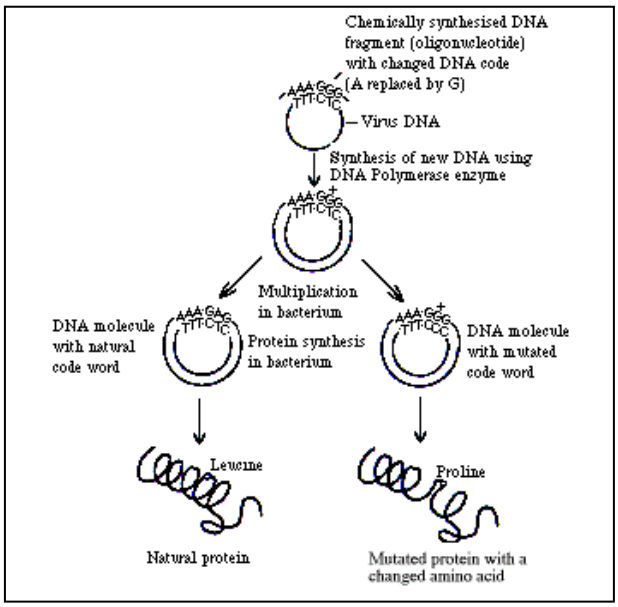


Fig 1: The principle of site-directed mutagenesis

The mutagenic oligonucleotides annealed the DNA template and served as a primer for DNA replication in the presence of the enzyme *DNA polymerase*.

The resulting product from DNA replication was a double-stranded DNA molecule that contained the point mutation derived from the mutagenic oligonucleotides.

The double-stranded DNA was circularized using the enzyme DNA ligase, separated from the residual template, and introduced into a suitable host organism for multiplication, selection, and phenotypic observation.

Reportedly, the method efficiency depended on removing non-mutagenized molecules and incomplete DNA duplexes before introducing them into the host organisms for multiplication.

- **Polymerase Chain Reactions (PCR)-Based Site-Directed Mutagenesis**

The principle used in the mutagenesis of a circular single-stranded DNA template for introducing a predefined point mutation can be applied, with some modifications, to introduce deletion, insertion, and point mutations to circular and linear double-stranded DNA templates.

Nonetheless, the earlier site-directed mutagenesis methods were low in efficiency, laborious, and time-consuming. To increase the method efficiency, researchers focused

on the strategy used in selecting the mutated DNA molecules and removing those that were not mutated.

The invention of polymerase chain reaction in the late 80s has enabled researchers to address the efficiency of this technique using another approach. Instead of focusing on the post-mutagenesis process, researchers can shift the focus directly to the mutagenesis process.

- **Can PCR Create Multiple Copies of a Specific DNA Fragment *In Vitro*?**

PCR is an *in vitro* technique that creates multiple copies of a designated region on the DNA template. It uses *a pair* of oligonucleotides as primers to bind with the template on opposite DNA strands and *deoxynucleic triphosphates* as building blocks to synthesize new DNA strands in the presence of a thermostable DNA polymerase enzyme. The newly synthesized DNA strands serve as templates in the following PCR rounds, thus the term *chain reactions*.

PCR consists of three steps: *denaturation*, *annealing,* and *extension*. Each step is performed only for a few seconds at different temperatures after the other in a cycle.

Theoretically, the number of the targeted DNA fragments increases twofold after the three steps are complete. After several PCR cycles, $2^{number\ of\ cycles}$ PCR products are produced from the PCR reaction.

- **Reactions of PCR-Based Site-Directed Mutagenesis Process**

PCR is integrated into site-directed mutagenesis via the use of oligonucleotides containing the designated mutations. Only one or both PCR primers act as mutagens for site-directed mutagenesis.

The following reactions take place during PCR-based site-directed mutagenesis:

1. In the *denaturation step*, high temperature breaks the hydrogen bonds between the double-stranded DNA template, separating the strands.
2. During the *annealing step*, the mutagenic oligonucleotides bind to complementary nucleobases on one strand. The other oligonucleotides bind to the complementary nucleobases on the opposite strand.
3. In the *extension step*, new daughter strands are synthesized by the enzyme DNA polymerase, extending the primers.
4. At the end of the extension step, the resulting two complementary daughter strands possess the mutations derived from the mutagenic primers and serve as templates in the subsequent PCR cycle, in addition to the original template.

In vitro mutagenesis

Another use of cloned DNA is *in vitro* mutagenesis in which a mutation is produced in a segment of cloned DNA. The DNA is then inserted into a cell or organism, and the effects of the mutation are studied. Mutations are useful to geneticists in enabling them to investigate the components of any biological process. However, traditional mutational analysis relied on the occurrence of random spontaneous mutations—a hit-or-miss method in which it was impossible to predict the precise type or position of the mutations obtained. In vitro mutagenesis, however, allows specific mutations to be tailored for type and for position within the gene. A cloned gene is treated in the test tube (in vitro) to obtain the specific mutation desired, and then this fragment is reintroduced into the living cell, where it replaces the resident gene.

One method of in vitro mutagenesis is oligonucleotide-directed mutagenesis. A specific point in a sequenced gene is pinpointed for mutation. An oligonucleotide, a short stretch of synthetic DNA of the desired sequence, is made chemically. For example, the oligonucleotide might have adenine in one specific location instead of guanine. This oligonucleotide is hybridized to the complementary strand of the cloned gene; it will hybridize despite the one base pair mismatch. Various enzymes are added to allow the oligonucleotide to prime the synthesis of a complete strand within the vector. When the vector is introduced into a bacterial cell and replicates, the mutated strand will act as a template for a complementary strand that will also be mutant, and thus a fully mutant molecule is obtained. This fully mutant cloned molecule is then reintroduced into the donor organism, and the mutant DNA replaces the resident gene.

Another version of *in vitro* mutagenesis is gene disruption, or gene knockout. Here the resident functional gene is replaced by a completely nonfunctional copy. The advantage of this technique over random mutagenesis is that specific genes can be knocked out at will, leaving all other genes untouched by the mutagenic procedure.

Gene knockout

A knockout refers to the use of genetic engineering to inactivate or remove one or more specific genes from an organism. Scientists create knockout organisms to study the impact of removing a gene from an organism, which often allows them to then learn something about that gene's function.

Gene knockout is the complete elimination of genes from an organism. Gene knockdown is the reduction of the expression of a gene in an organism. It can happen only by genetic engineering techniques.

Process of gene knockout:

A typical process of KO involves steps like– gene *selection, Vector design* and *construction, transformation, Homologous recombination, selection, identification* and *validation, insert validation, animal model generation* and *phenotypic assays*.

We can consider gene KO as one kind of genetic engineering experiment.

- ✓ **Gene selection:**

The very first step in KO is selecting a gene we want to knock out. A gene is selected only if its function is known and should have been involved in any biological activity or biological process we wish to study.

Some dry lab work has been carried out to understand the structure of a gene. It is mapped on a chromosome and other parameters such as gene length, No of exons and introns, promoter length and sequence, etc are closely examined. Dry lab work helps construct an effective vector for the experiment.

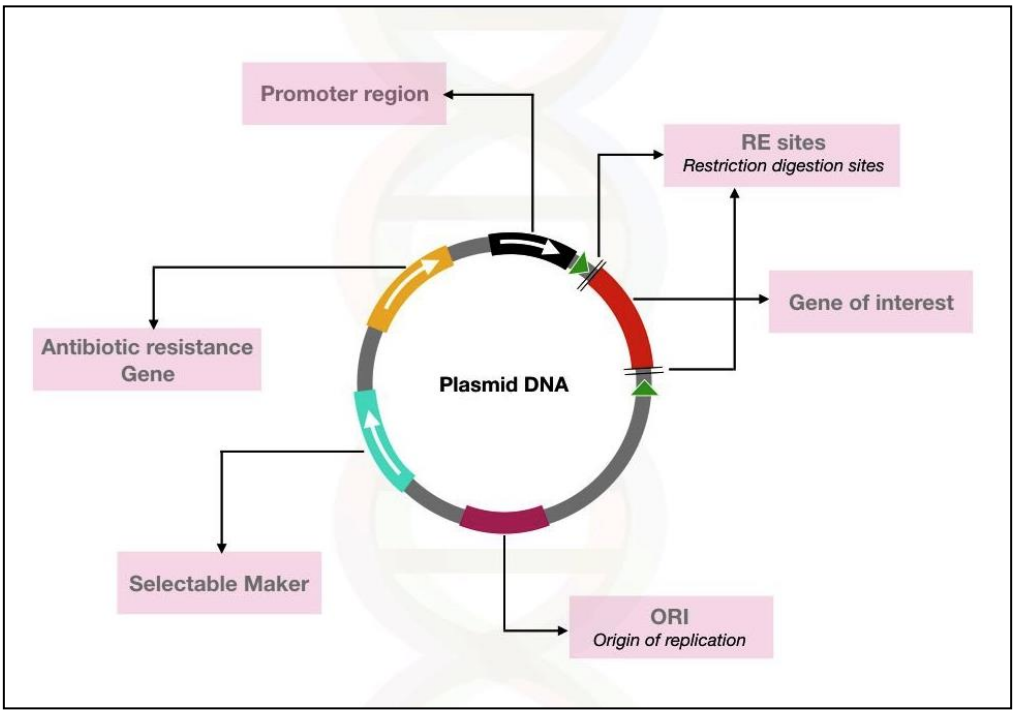


Fig 2: A general structure of a plasmid used in genetic engineering experiments

- ✓ **Vector design and construction:**

A vector is a vehicle that carries our genetic material and transfers it to another location. Viral vectors, plasmids, cosmids and bacterial artificial chromosomes are a few common types of vectors used in gene therapy experiments.

Among all these, a plasmid is the most popular and common vector used in KO. The plasmid is bacterial extrachromosomal and circular DNA that can carry the target mutation or sequence for KO.

A vector consists of the selected marker, a gene-specific homologous region, and a disrupting element- a mutation or sequence. After vector construction and validation, our plasmid vector is ready for transformation.

- ✓ **Transformation:**

Now, our plasmid is ready to insert into a target cell. Scientists usually select Embryonic Stem Cells (ESCs) for transformation. ESCs can divide at a faster rate into different cell types and form various tissues.

The vector once finds the gene-specific target location, it recombines and inserts our target 'change' with the marker gene in the target cells. Transformed cells are then cultured using an appropriate media.

Electroporation, Sonication and micro-injection are some common techniques for ESCs transformation. Note that cells will only grow if transformation occurs.

- ✓ **Homologous recombination:**

After successful transformation, and after reaching the complementary region, homologous recombination will occur which results in the disruption of a gene's function later on. HR introduces the 'change' that eventually knocks out the target gene.

- ✓ **Selection and validation:**

Now, it's important to validate whether the transformation fully occurred or not. Meaning, the experiment is successfully placed or not. Techniques like polymerase chain reaction and DNA sequencing can validate KO.

A few transformed cells are taken for DNA isolation. DNA sequencing can validate KO at the sequence level. PCR and qPCR can also be used for validation using sequence-specific primers.

qPCR can determine the concentration of the target gene from the transformed cells and determines if knockout occurred or not. Besides, restriction digestion can also be employed for KO verification but is less recommended.

After the completion of validation, the successfully transformed cells are selected for the generation of genetically modified model organisms.

- ✓ **Introduction into the animal model:**

Now the transformed cells are employed to generate a KO animal using breeding and other techniques.

- ✓ **Phenotypic investigation:**

Phenotypic analysis includes physical, biochemical and functional investigations. *In vitro*, experiments are carried out to study the effect of gene loss. Various chemical assays are also available for protein studies as well.

The entire process of gene knockout is represented in the figure below,

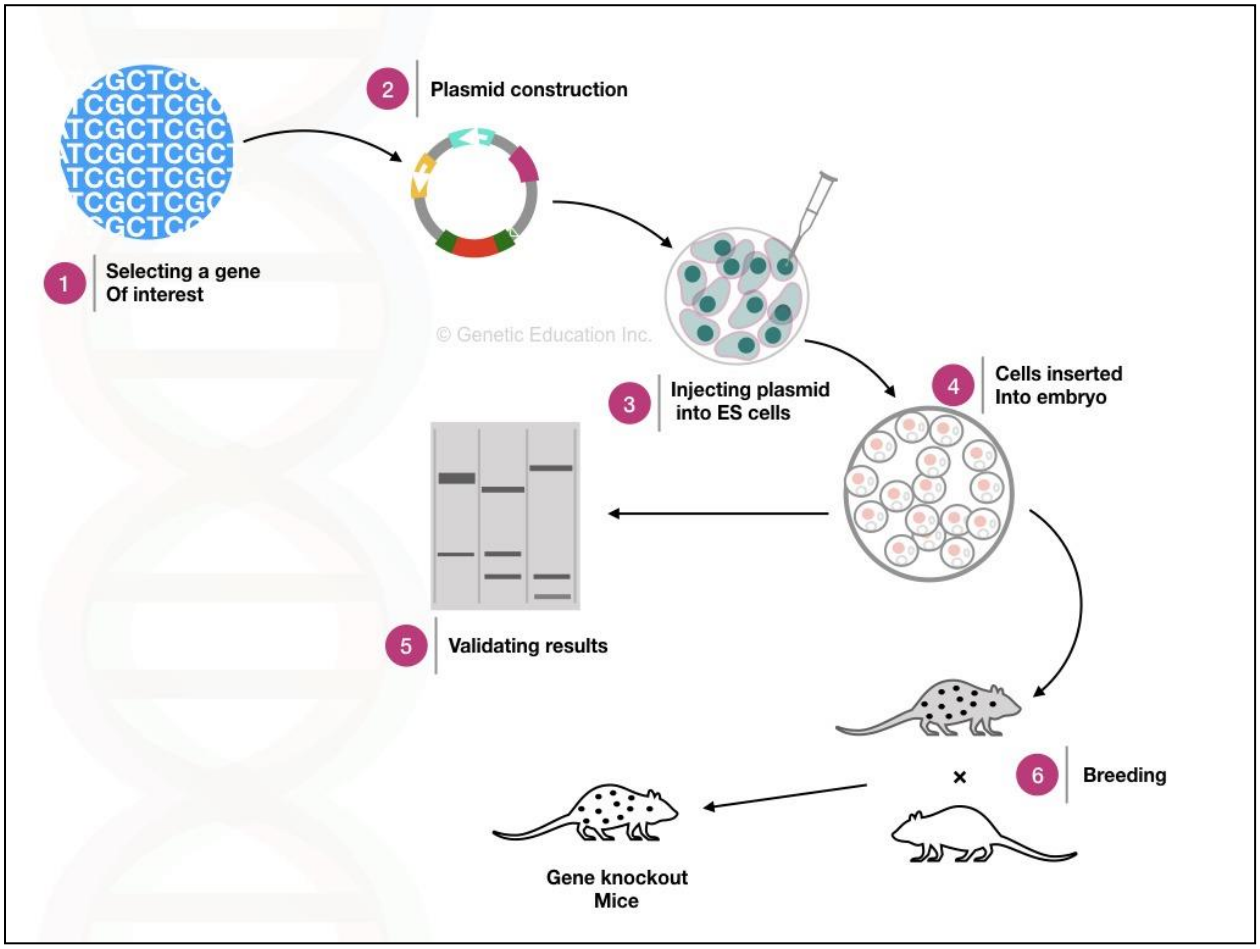


Fig 3: Entire gene knockout method

Gene knockout Methods:

So far we have discussed the KO experiment in context with the homologous recombination, however, there are other methods as well those scientists can use. Gene silencing, conditional knockout, gene editing and knockout by mutation are a few common methods for KO. We will discuss each technique one after another in this section. But remember, in any method, the purpose is to inactivate or make silence the target gene *viz* knockout.

- ✓ **Conditional knockout:**

Conditional KO is 'specific.' It allows the inactivation of a target gene in a specific tissue or specific developmental stage. Or gene inactivation has been carried out to study a specific function in a specific cell population, tissue or developmental stage.

Conditional KO is a great tool to study tissue-specific cancer. The present technique uses homologous recombination and tissue-specific promoter for conditional KO where

knockout only occurs in selected tissues or cells. CKO is the best way to study a gene's function at a tissue or cellular level.

Interestingly, it is not only performed in the embryonic stage but also on adult animals as well. Cre-LoxP is one such technique in which the "Cre" recombinase works on LoxP tissue-specific inactivation. Recent studies showed that CKO is widely used in breast cancer and ovarian cancer research.

Conditional knockout is very popular and by far the most successful technique for gene inactivation. We will cover the whole topic in some other article.

- ✓ **Gene knockout by mutation:**

KO by mutation is yet another important technique. In this technique, a mutation is incorporated in a target gene or sequence in a way that deactivates or alters the target gene's function.

Such a mutation is known as loss of function mutation which can be either insertion, deletion, duplication or translocation. Artificial mutagenesis techniques like chemical alteration, radiation-induced mutagenesis or physical mutagenesis have been used to incorporate a mutation.

Notedly, artificial mutagenesis induces mutation in a gene's coding, promoter or any regulatory region to knock it out.

- ✓ **Gene knockout by gene therapy:**

Knockout by gene therapy is a novel approach, which involves the delivery of a therapeutic agent, engineered vector or system to suppress a gene's function.

CRISPR-CAS9 is a popular and modern gene therapy technique that can introduce a mutation or remove a sequence from the target gene. Any target element, for example, the sgRNA in the case of the CRISPR technique, is engineered in a way that introduces a desired change in the DNA sequence.

Such techniques are very accurate and precise for gene knockout. Gene therapy approaches hold promise for potential applications in treating genetic diseases. If you would like to learn more, you can read these articles.

- **Homologous Recombination:**

Homologous recombination involves the exchange of genetic material between two homologous sequences or genes. Homologous recombination has a higher success rate. Thus, Scientists use this phenomenon for KO.

Here, scientists prepare homologous sequences complementary to the flanking region of the target gene and introduce them in the vector. The vector transfers it to the target location, where the recombinase enzyme performs recombination and exchanges the native sequence with our target sequence.

Interestingly, this process either disrupts a gene's function, eliminates it or suppresses it completely. Notedy, as aforesaid, a marker gene is also incorporated for validation.

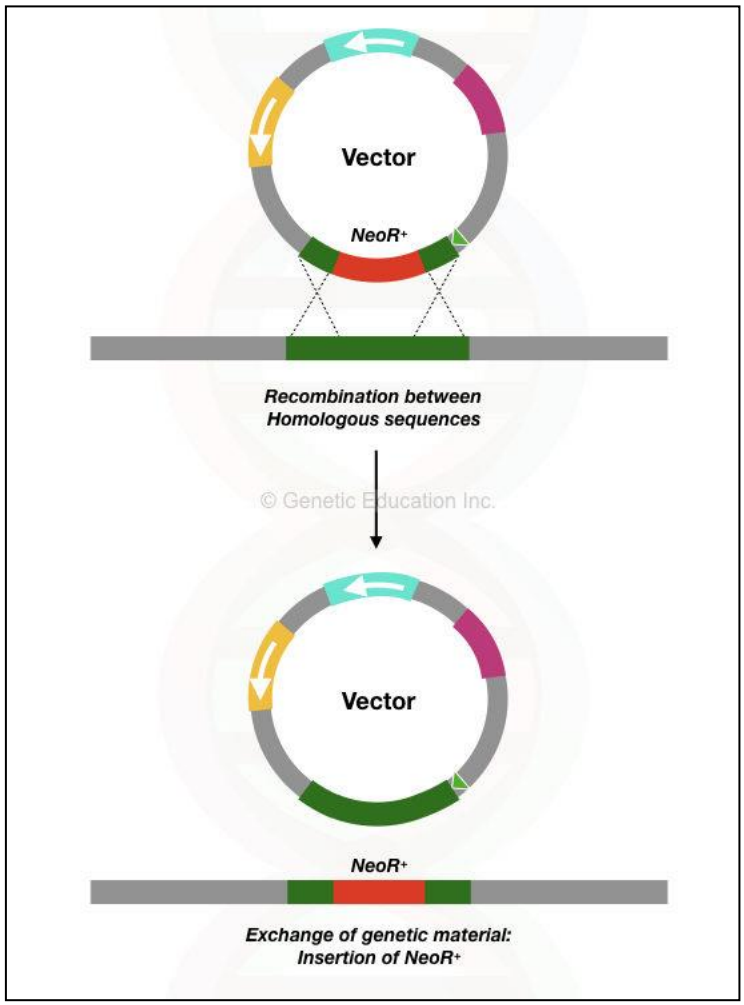


Fig 4: Hypothetical representation of recombination event between the plasmid and target gene.

Applications of Gene Knockout:

The key function of KO is to eliminate or inactivate a gene. Thus, making it functionally inactive. The present revolutionary approach has opened new doors for researchers to study diseases, genes and traits. Here are some of the amazing and important applications of gene knockout.

1. **Understanding a gene's function:**

Discussion so far demonstrates that the pivotal function of KO is to understand a gene's function. Scientists can study alterations in phenotypes or traits by selective knockout or target gene inactivation. KO experiments on model organisms help study genes' role in development, behavior, biological activities, physiology and disease progression. Eventually, such studies contribute to develop new strategies and therapies for diseases.

2. **Genotype and phenotype relation:**

Gene knockout is a great tool to establish the relationship between a gene and a phenotype. Through selective gene inactivation, its effect on a phenotype or group of phenotypes can be studied. This way, scientists understand the relationship between a gene and a phenotype.

3. **Functional genomics:**

Functional genomics is largely defined as 'to understand the overall function of the genome.' KO is a crucial tool in functional genomic studies. Through selective gene targeting, scientists can systematically investigate the impact of individual genes on related functions, pathways, cellular activities, signaling pathways, etc.

Scientists can understand the function as well as the evolutionary importance of homologous genes and gene families too.

4. **Evolutionary studies:**

Gene knockout is also an amazing tool for studying Evolution and the role of a gene in evolutionary events. By selectively inactivating a gene across different organisms, scientists can understand a gene's effect and its evolutionary importance. This helps to understand the underlying mechanism of evolution and biodiversity.

5. **Disease studies:**

KO also helps understand the molecular mechanism behind diseases through gene-level analysis. Scientists prepare animal models to perform KO for a particular disease-governing gene and understand its effect.

These studies help us understand how genetic diseases happen and how certain genes or groups of genes play a role in causing disease. This understanding helps in creating new treatments for the disease.

6. **Drug studies and development:**

Assessing a disease at the molecular level and using gene knockout techniques also aids in understanding drug responses. This means that scientists can evaluate the effectiveness, efficiency, and response of a drug when targeting specific genes or when those genes are not targeted.

Such valuable information provides value in drug development, studies and discovery. It also strengthens the therapeutic approach against a disease.

7. **Developing gene therapy:**

Gene knockout recently has given more strength to the field of gene therapy. New therapeutic approaches and gene therapy treatments can be developed against particular or many gene defects.

Various CRISPR-cas9 therapies mediated by KO are now under clinical trials and ready to rule the world. Other therapies are also under research.

8. **Agriculture and livestock improvement:**

Not only in disease have studies and human genetics, but gene knockout techniques also had great value in the agriculture industry and livestock improvement. Through knockout studies, researchers can understand the function of an economically important trait and thereby can enhance it.

Desirable and economically important traits like disease-resistant, yield improvement, nutrient values in plants and growth, development and milking capacity in animals can be improved.

Limitations of Gene Knockout:

Gene knockout is a highly useful and precise tool in the field of genetic engineering. However, like other techniques, it also has certain important limitations. Here are some limitations of gene knockout (KO):

1. **Off-targeting effects:**

Any gene knockout experiment is designed to specifically inactivate a target gene, however, often times, it can suppress or affect other gene's activity, unintentionally. Or in other cases, it inactivates entirely a different gene. These two scenarios are known as an off-targeting effect.

Research shows substantial off-targeting gene knockout effects, which may directly or indirectly impact the function of other gene(s) or regulatory elements.

2. **Gene compensatory effects:**

Compensatory mechanisms may exist for several genes that compensate or fill the function of an inactivated gene. This particularly occurs during the developmental process in which KO triggers the developmental compensatory mechanism.

Resultantly, even after successful gene knockout, the function will remain intact. Such events pose challenges to understanding the full potential effect of a gene and its governing traits.

3. **Compensatory action:**

In some cases, redundant genes can compensate for the impact of a target gene. Therefore, simply knocking out a single gene may not result in a loss of function. Other genes, known as redundant genes, can step in and continue to produce the expected traits. These compensatory effects of genes can restrict the effectiveness of gene knockout.

4. **Gene knockout for essential genes:**

There are a set of genes in organisms that are essential for their survival. Alteration in such genes can cause lethal effects or genetic abnormalities. Scientists can't manipulate and study these genes by gene knockout.

5. **Tissue specificity:**

Gene knockout has less tissue specificity as it can impact a broad range of cells and tissues. So while targeting a specific gene for specific tissues, adequate results can not be obtained.

Probable questions:

1. What do you mean by mutagenesis?
2. Describe the principle of site-directed mutagenesis with diagram.
3. Can PCR Create Multiple Copies of a Specific DNA Fragment *In Vitro*?
4. Describe the steps of Reactions of PCR-Based Site-Directed Mutagenesis.
5. Write short notes on *In vitro* mutagenesis.
6. What is Gene knockout? Name the processes by which gene knockout is done.
7. Discuss the process of gene knockout with diagram.
8. Define **Homologous recombination**.
9. Discuss the method of gene knockout.
10. Discuss the **Applications of Gene Knockout**.
11. What are the limitations of gene knockout process?

Suggested readings:

1. Snustad D P, Simmons MJ. 2009. Principles of Genetics. V Edition. John Wiley and Sons Inc
2. Strickberger M. W – Genetics; Macmillam
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The study materials of this book have been collected from books, various e- books, journals and other e-sources.