PRACTICAL MANUAL

IMMUNOLOGY

&

ANIMAL BIOTECHNOLOGY

D.N.R.COLLEGE(Autonomous),Bhimavaram

(AffiliatedtoAdikaviNannayaUniversity)

Semester-IV

II B.Sc. Zoology Practical syllabus (w.e.f 2020-21) admitted batch

Paper V - IMMUNOLOGY AND ANIMAL BIOTECHNOLOGY

Total Hours-24 **Hours per week-02** **Total credits-01**

I. Immunology

1. Demonstration of lymphoid organs (as per UGC guidelines)

2. Histological study of spleen, thymus and lymph nodes (slides)

3. Blood group determination

4. Demonstration of

   a. ELISA

   b. Immunoelectrophoresis

II. Animal biotechnology

1. DNA quantification using DPA Method.

2. Techniques - Western Blot, Southern Hybridization, DNA Fingerprinting

3. Separation, Purification of biological compounds by paper, Thin-layer and Column chromatography

4. Cleaning and sterilization of glass and plastic wares for cell culture.

5. Preparation of culture media.

D.N.R. COLLEGE (AUTONOMOUS), BHIMAVARAM

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II B.Sc.Zoology practical Examination

Semester-IV

Paper V - IMMUNOLOGY AND ANIMAL BIOTECHNOLOGY

Model question paper and scheme of valuation

(w.e.f 2020-21 admited batch)

Duration :3 hrs Max.Marks:50

1.Identify the following slides 3x5=15M

A) B) C)

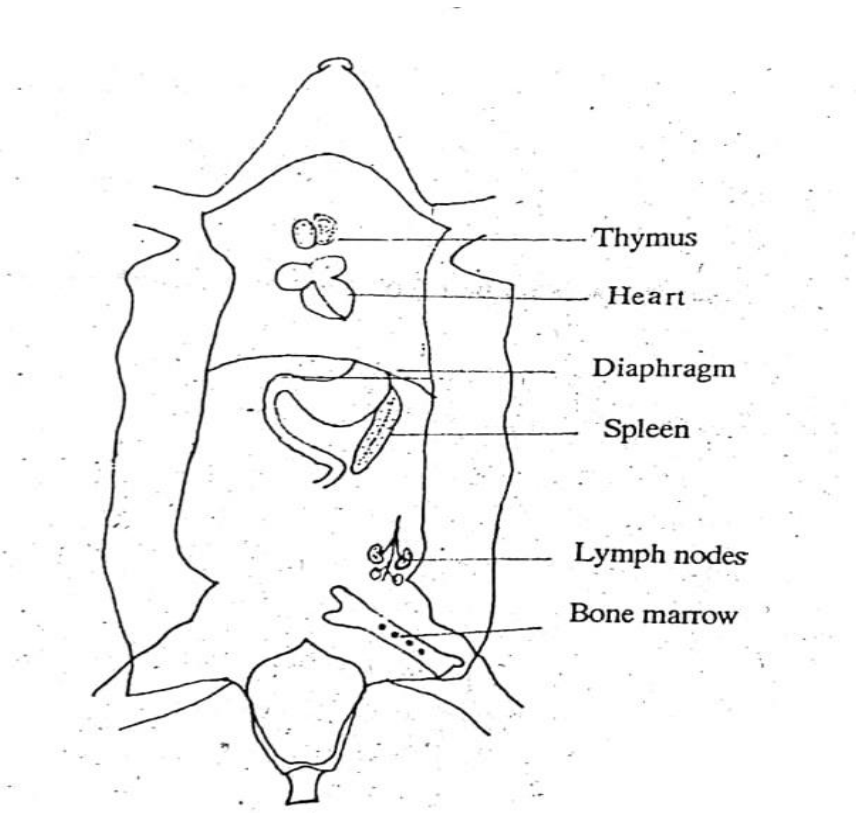
2.Identify the blood group in the given sample 10M

3.Write briefly about DNA fingerprinting technique 15M

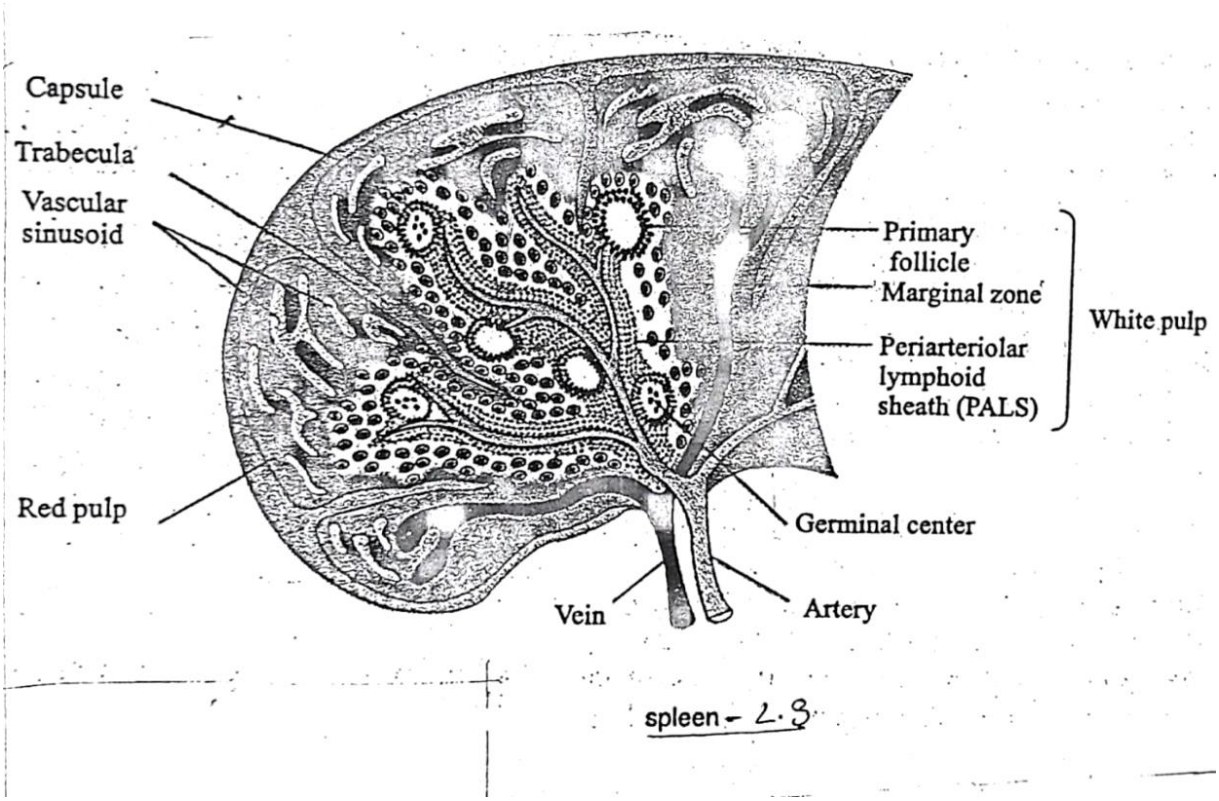
4.Viva voice 05 M

5.Record 05 M

LYMPHOID ORGANS IN RAT



L.S. OF SPLEEN


Capsule
Trabecula
Vascular sinusoid
Primary follicle
Marginal zone
Periarteriolar lymphoid sheath (PALS)
White pulp
Red pulp
Germinal center
Vein
Artery
spleen - L.S

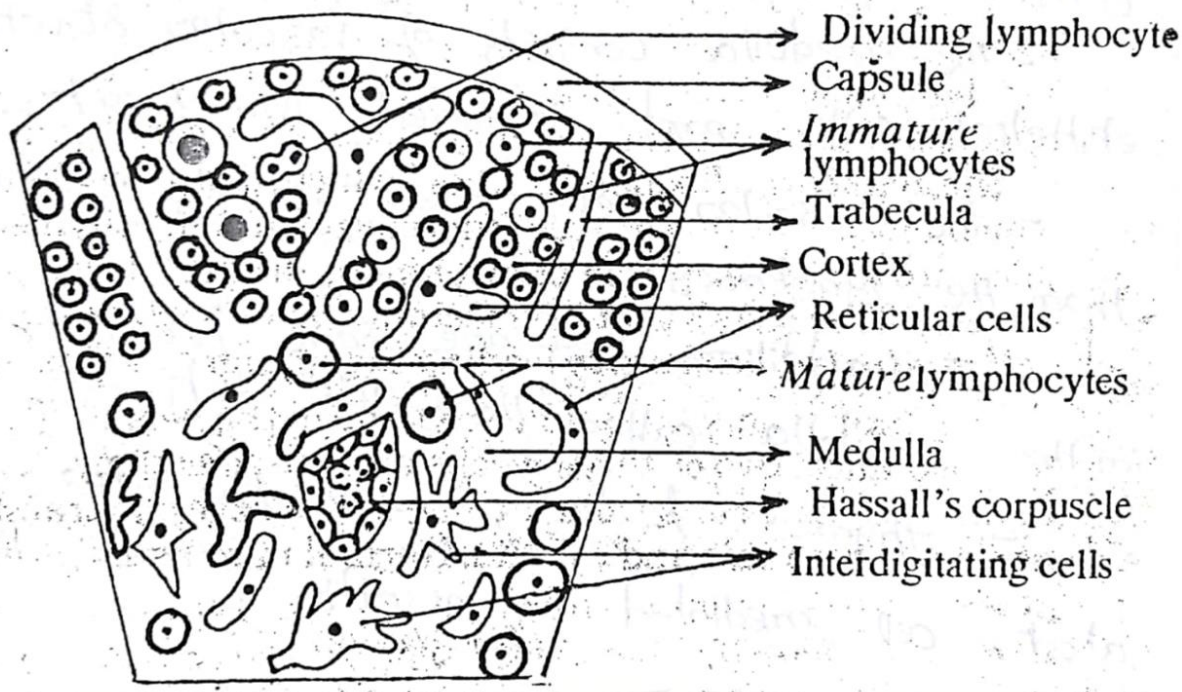
L.S. OF SPLEEN

- Spleen is a secondary lymphoid organ located in the upper abdominal cavity behind the stomach are close to the diaphragm.
- It is deep red in colour.Spleen is surrounded by a capsule. The capsule Penetrates into the tissues as septa called trabeculae.
- There are two distinct regions in the spleen, namely outer red pulp and inner white Pulp.
- Red pulp consists of large numbers of blood filled sinusoids in which phagocytes and plasma cells are found.
- The red pulp is also reserve site for haemopoiesis.
- The white pulp consists of the lymphoid tissue.
- The major Part of lymphoid tissue is arranged around a central arteriole and it is known as Periarteriolar lymphoid sheath. It consists of B-lymphocytes grouped into Small masses called follicles. In between follicles T lymphocytes are distributed.
- The B lymphocyte follicle may also contain germinal centre, it is known as Secondary follicle.
- The Periarteriolar lymphoid sheath is separated from the red pulp by marginal zone consists of both T and B lymphocytes.

Functions:

It is graveyard for worn out blood cells. A reserve tank in the formation of RBC

T.S. OF THYMUS

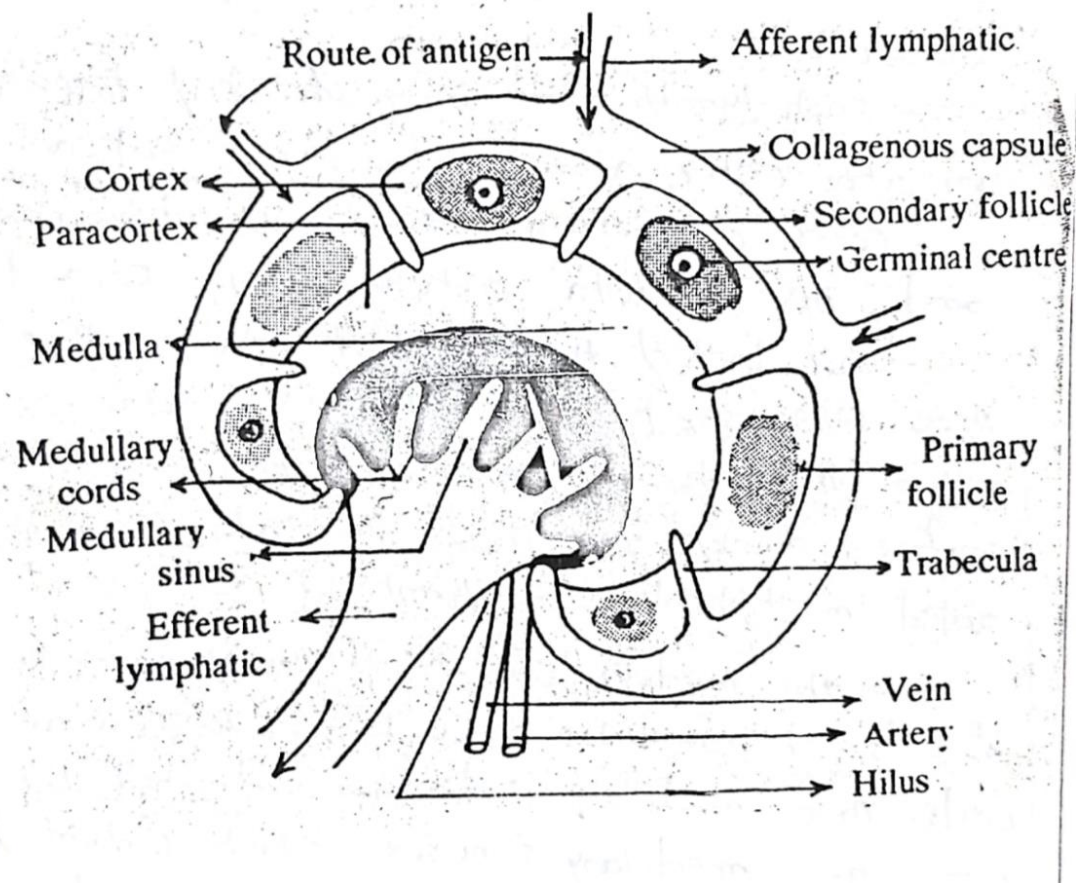


Section of thymus

T.S. OF THYMUS

- Thymus is a Primary lymphoid organ.
- In humans, the Thymus occur as two oval lobes Just behind the top of sternum,below the thyroid gland.
- Thymus is covered by a fibrous capsule. It is formed of two lobes. The cells are arranged into an outer cortex and inner medulla.
- The cortex consists of lymphocytes and reticular cells.
- The medulla consists of vascular structure, reticular epithelial cells and scattered lymphocytes.
- The reticular cells are relatively more in number than the lymphocytes.
- In addition there are some peculiar structures in the medulla called Hassall's corpuscles.
- Thymus produces T-lymphocytes and it brings about cell mediated immunity.

L.S. OF LYMPH NODE



L.S. OF LYMPH NODE

- Lymph nodes secondary lymphoid organ.
- Human lymph nodes are bean or kidney shaped.
- One Side the lymph mode has an indentation, the hilus, where the blood vessels enters and leave the node.
- Several afferent lymphatic ducts carry lymph into the gland and a single efferent lymphatic duct leaves the node.
- The lymph node is covered by a capsule. The capsule Penetrates into the lymph node to from septa called trabeculae.
- Each lymph node is made up of three regions namely an outer cortex, a middle paracortex and an inner medulla.
- Cortex contains B cells, paracortex contains T cells and medulla contains both the B and T cells.
- The bulk of the lymphoid cells is present in the cortex.These cells group together to form nodules or Primary follicles.With in these primary follicles, secondary follicles with germinal centers developed.The germinal centers are the sites of rapid multiplication of lymphoid cells.
- The medulla consists of lymphocytes arranged along strands of Connective tissue fibers known as medullary cords. These medullary cords are separated by large sinuses known as medullary sinuses which contain plasma cells.
- The cortical and medullary regions form the bursa dependent or B dependent areas. The Para cortical area forms Thymus dependent or T dependent area.

DETERMINATION OF BLOOD GROUPS

AIM:

To determine the blood groups in the given sample.

PRINCIPLE:

ABO blood group system was first discovered by Landsteiner in the year 1900. Determination of blood groups is essential mainly for blood transfusion,paternity test and other medico-legal problems.

The R.B.C contains two types of antigens. They are antigen-A and antigen-B.The plasma contains the corresponding antibodies calledAntibody-'a'and Antibody-'b'. Based on the presence or absence of antigens and antibodies human blood is classified into 4 groups called A,B,AB& O.

A-group persons contain Antigen-A & AntibodyB.

B-group persons contain Antigen-B&AntibodyA.

AB-group persons contain both AntigenA & Bbut noAntibody.

O-group persons contain no Antigen but both antibodies A &B

are present.

To determine the blood groups the R.B.C are made to react with sera containing known antibody with antigen present in theR.B.C.They will be either agglutination or no agglutination.

APPARATUS:

Glass slides,disposable,needle, cotton, microscope.

REAGENTS:

Anti-A blood group serum - Blue colourAnti-B blood group serum - Yellow colourAnti-Dblood groupserum - colourless

PROCEDURE:

Clean the glass slide with water. Clean the finger tip with spiritand allow it to dry. The fingertip is pricked with needle. 3 drops of blood is collected on a glasss lid seperately.To this AntiA,B,D are added respectively.The dropper should not touch the blood drop on the slide.The contents are gently mixed by using another glass slide corner. Slowly till the slide from side to side for few minutes. The slide is kept undisturbed for 5-10 minutes and the blood groups are identified.

OBSERVATION:

If Agglutination occurs with Anti-Aserum,the tested bloodbelongs to 'A' group. If agglutination occurs with Anti-b serum the tested bloodbelongsto'B'group.If agglutination occurs with both A&B sera the blood group is AB. If agglutination is not seen in any Anti-serun then it is groupis'O'.If agglutination occurs with Anti-'D'the blood belongs to Rh(+ve),if no agglutination the blood is Rh(-ve).

RESULT:

The blood group of the given sample of blood is identified as .

ENZYME- LINKED IMMUNOSORBENT ASSAY(ELISA)

- ELISA is an immunological technique used for the qualitative produced by determination of the concentration of certain antigens or antibodies.
- The ELISA technique was first introduced in 1970 by Engvall and Perlmann.
- The antibodies are absorbed to a solid support,a polystyrene microtiter plate.
- The support after coating with antibody is washed.

- The antigen is added and it binds to the absorbed antibodies. Then an enzyme-linked antibody molecule called the conjugate is added which also binds to the antigen.
- A chromogenic substrate for the enzyme is added and the coloured product generated is measured. The intensity of the colour is proportional to the bound enzyme and thus to the amount of the bound antigen.
- Hence the intensity of the colour produced by a series of standard antigens allows the calculation of the amount of antigen in an unknown sample.
- ELISA is used to detect a variety of antibodies and antigens such as plant and animal hormones, toxins and viruses.
- It is a successful technique for detecting AIDS.
- ELISA is very sensitive,safe to use and less costly.

There are a few variant so ELISA to detect antigens and antibodies in the sample.They are,

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. CompetitiveELISA

Direct ELISA:

Direct ELISA is also called simple ELISA. In this method,antigens are coated in a microtiter well and then enzyme-linked antibody is added to the well. Then the substrate is added to the well. The colour change is noted.

-This method helps to determine the presence of supposed antigen in samples.

-It is used in agriculture,medicine and in industries

Sandwich ELISA:

- In Sandwich ELISA,the antigen is held between the antibodies.
- The microtiter well is coated with a known quantity of antibody.
- The sample antigen is applied on the titer well.
- The well is washed to remove unbound antigens.
- A specific antibody is added.
- The specific antibody binds to the antigen.
- The antigen is held between the antibodies. Hence the name sandwich ELISA.
- Enzyme-linked secondary antibodies are added as detection antibodies.
- They bind to the FC region of specific antibodies.
- The well is washed to remove unbound enzyme linked antibodies.
- The substrate is added.
- The enzyme reduces the substrate to a coloured product.
- The intensity of colour is measured.
- The quantity of antigen in the sample is directly proportional to the intensity of colour.

Applications of ELISA:

- ELISA is used as a screening test for HIV.

- It helps to detect HIVantibodies in theblood.
- It helps to detect mycobacterium antibodies in tuberculosis.
- Ithelps to detectrotavirus in thefaeces.
- It helps to detect hepatitisis in the serum.
- It helps to detect enterotoxin of E.coli in the faeces.

IMMUNOELECTROPHORESIS

The separation of antibodies in an antiserum by combining the techniques of gelelectrophoresis and double immuno diffusion of gel electrophoresis and double immuno diffusion is called immune electrophoresis. This method was advised by Grabar andWilliams1953.

Immunoelectrophoresis has two major steps.They are;

-Gel electrophoresis

-Double immunodiffusion

Gel electrophoresis.

This is the first step of immune electrophoresis.

The proteins and antibodies in the antiserum are separated into separate bands based on their size and charge under the electricfield.

Steps involved in gel electrophoresis ;

- Proteins and antigens in a cell type or pathogen or human serum are isolated in a solution by adopting a standard procedure for protein isolation.
- 2 grams of agar is dissolved in 50ml of diethylbarbiturate buffer and 50ml of water and its PH is adjustedto8.2.This agar is melted by heating.
- The melted agar is poured on glass slide to a height of 3 mm.

- Then the slide is kept as such for 15 minutes in a moist chamber or petriplate to solidify the gel.
- A trough of 5 cm length and 1 mm width is cut length wise at one side of the gel. Further, a round well of 1 or 2 mm diameter is made just below the one end of the trough.
- The sample containing antigens is loaded into the well using a micropipette.
- One filter paper strip is inserted into either ends of supporting stage in the electrophoresis apparatus.
- Buffer tanks are filled with Tris-HCL buffer and electrodes are fixed in the tanks.
- The electrophoresis apparatus is closed.
- Electric current of 3-6 volts is applied for 45 minutes.
- During this time,antigens are separated based on their size and charge.
- After this time,the current is switched off and the agar plate is taken out to perform double immunodiffusion.

Double Immunodiffusion:

The antigens and antibodies are allowed to diffuse through the gel. As a result, the antigens and antibodies meet together between the well and trough. It leads to the formation of antigen-antibody complexes in the form of precipitin bands.

Steps involved in the procedure:

- Antiserum is prepared from rabbit's blood.
- The antiserum is loaded into the trough of agar plate containing separated antigens using a micropipette.
- The agar plate is kept in a petri dish containing moist cotton.

- The petriplate is incubatedat16-20℃ for about 20hrs.
- During this time,antigens diffuse from the separated bands and antibodies diffuse from the trough.They form Ag –Ab complexes where ever they meet together.
- After immune diffusion,the agarplate is washed with physiological saline to remove unbound antigens and antibodies.
- The agar plate is kept at37℃ for about15 minutes to remove water from it.
- Then it is stained with amido black or light green Sudan black dye for1hour.
- It is wasted with 50% ethanol to remove unbound dye components.
- The agar plate is photographed. It can be visualized under a microscope for precipitin bands.

USES:

- Immuno electrophoresis is used to determine precipitating antibodies in antiserum and respective antigens in the serum sample.
- In industries,it isused to test the purity of antibodies and r-DNA products.
- In plant sciences,abnormal proteins in a diseased specimen related to a disease free specimen can be determined.
- In clinics,it is very useful to determine normal and abnormal serum proteins.Concentrated urine, spinal fluid and patient’s serum are used as samples.

1. ESTIMATION OF DNA BY DIPHENYLAMINE REACTION

Principle:

The deoxyribose in DNA in the presence of acid forms β-hydroxylevulinaldehyde which reacts with diphenylamine to give a blue colour with a sharp absorption maximum at595nm. In DNA, only the deoxyribose of the purine nucleotides react, so that the value obtained represents half of the total deoxyribose present.

Equipment:

Spectrophotometer and water bath

Chemicals/reagents:

Standard DNA solution (0.25mg/ml),Diphenylamine reagent DNA sample in saline citrate buffer, Saline citrate buffer (0.15M NaCl, 0.015M sodium citrate, pH 7.0) Glacial acetic Acid , Concentrated H_2SO_4, Ethanol.

Glassware:

Test tubes, Pipettes ,Graduated cylinder.

Preparation of reagent:

Dissolve 1.5g diphenylamine in 100ml of glacial acetic acid. Add 1.5ml of conc H2SO4. Store the solution in a dark glass bottle. On the day of use, prepare a fresh solution of ethanol (1ml) in dH2O (50ml). Add 0.5ml of this solution to each 100ml of the diphenylamine solution.

Assay:

1. Prepare a series of dilutions of standard DNA (0.25mg/ml) in saline citrate buffer to give a concentration of 50-500µg/ml.

2. Prepare all the samples in triplicate.

3. To 2ml of each dilution of blank, standard and unknown add 4ml of diphenylamine reagent and mix. Tube-1 is used as blank and tubes 2 through 7 are used for construction of a standard calibration curve for DNA. Tubes 8-11 are for unknown samples. (Table1)

4. Incubate all the tubes in boiling water for 10 min.

5. Cool the tubes and read the absorbance at 595nm against the blank.

6. Construct a standard curve of absorbance A595 vs. quantity of DNA and then calculate the concentration of unknown DNA dissolved in the saline citrate solution.

S. No	**DNA**		**DH2O**	**Reagent**	**A 595**
	µl	**µg**	**µl**	**ml**	
1	-	-	2000	4	
2	200	50	1800	4	
3	400	100	1600	4	
4	800	200	1200	4	
5	1200	300	800	4	
6	1600	400	400	4	
7	2000	500	-	4	
8	Unknown A	-	-	4	
9	Unknown B	-	-	4	
10	Unknown C	-	-	4	
11	Unknown D	-	-	4	

Calculation:

Determine the amount of DNA in the unknown sample by plotting a standard

curve of A595 on Y-axis and µg of DNA on X-axis.

2.SOUTHERN BLOTTING TECHNIQUE

Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labelling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc.

Blotting techniques are used to identify unique proteins and nucleic acid sequences. They have been developed to be highly specific and sensitive and have become important tools in both molecular biology and clinical research.

- Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample. The method is named after its inventor, the British biologist Edwin Southern.

- The procedure for Southern blot technique is as detailed below: Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments, which are then electrophoresed on an agarose gel to separate them by size.

- If the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (containing NaOH) to denature the double-stranded DNA.

- The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe and destroys any residual RNA that may still be present in the DNA.

- A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the

gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane.

 Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.

 The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.

 The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined.

 The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

 After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.

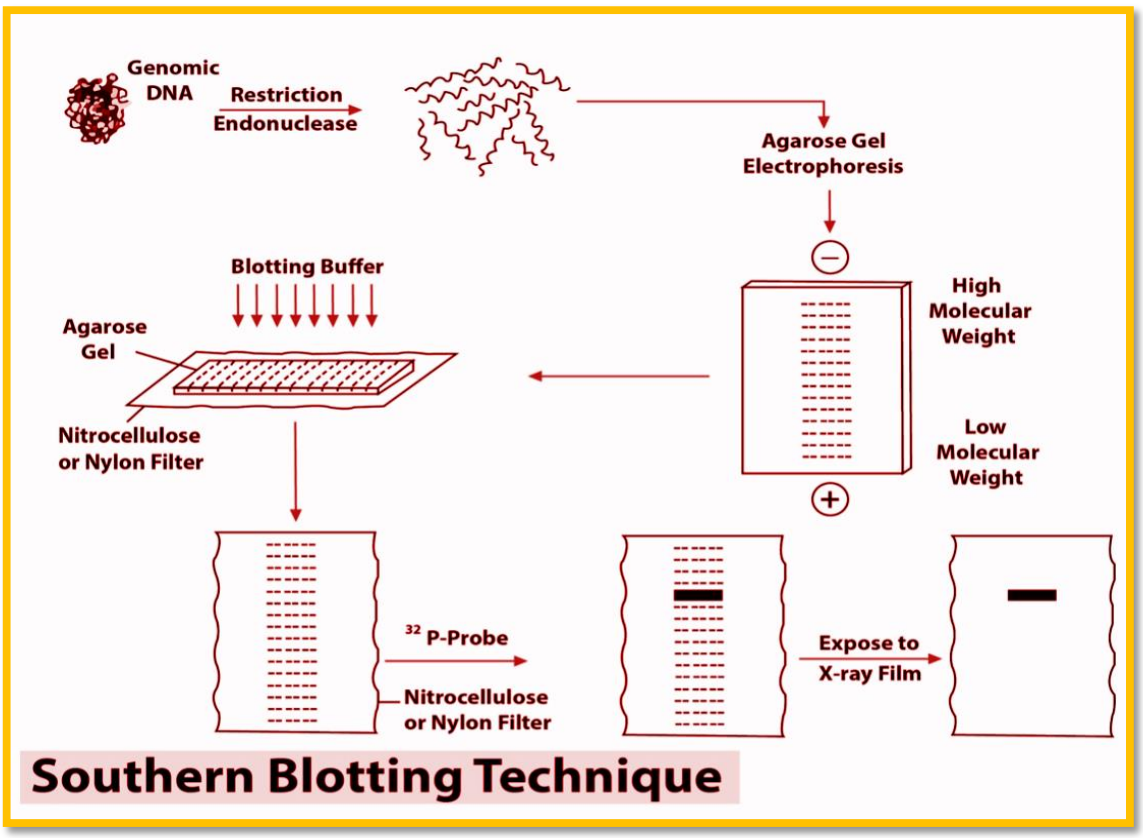
 Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe.

 The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labelled hybridization probe to the size-fractionated DNA. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome.

 A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas

multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication).

 Modification of the hybridization conditions (i.e, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.



3.WESTERN BLOTTING TECHNIQUE

Western blotting is a widely used technique for the detection and analysis of proteins based on their ability to bind to specific antibodies.. It was first described by Towbin, et.al in 1979 and has since become one of the most commonly used methods in life science research. The specificity of the antibody- antigen interaction enables to a target protein to be identified in the midst of a complex protein mixture.

It is an analytical method where in a protein sample is electrophoresis on an SDS-PAGE and electro transferred on to PVDF membrane or nitrocellulose membrane. The transferred protein is detected using specific primary and secondary enzyme labelled antibody First, proteins are separated from each other based on their size. Second, antibodies are used to detect the protein of interest. Finally, a substrate that reacts with an enzyme is used to view the antibody/protein complex.

Procedure-

Step-1 Sample Prepration

First step in the sample preparation is isolating the protein from a sample. Usually proteins are purified from cells.

Next the protein concentration is determined. Sample buffer commonly Laemmli buffer, which contains Sodium dodecyl Sulpate (SDS) and beta- mercaptoethanol (BME) is added to the protein suspension.

The sample buffer is the centrifuge and heated to near- boiling, which denatures the protein and allows the SDS to bind to the protein. SDS carries negative charge. Glycerol to make samples sink into wells and the Tris base provides appropriate pH. The blue dye to visualize samples as gel is run.

Step-2 SDS- PAGE

The first step in SDS-PAGE is placing the sample, containing the protein- SDS compound, in a well on top of the gel. A molecular weight marker is usually

loaded in one of the well, which determines the molecular weight of other proteins on the gel. And also the samples are added in the remaining wells. The protein size is measured in kilo daltons (kDa).Once the marker and samples are loaded, a current is run across the gel. With a negative pole on the well of the gel, a positive pole on the opposite end of the gel. Because the protein is bounded to negatively charged SDS, it is pulled down through the gel to the positive pole. The larger the protein, the slower it moves.Although the running the gel is the last step in the SDS- PAGE method, It is important to make note of several pieces of information.

•Proteins are separated by their size as they run through the gel.

•The lower the concentration of acrylamide in a gel, the easier it becomes for proteins to move through the gel , so all proteins move further under the same conditions.

•Gradient gels are where the concentration of acrylamide increases from the top to the bottom.

Step 3: Membrane Transfer (Wet Transfer)

After the gel is run, it is placed against a membrane , and current is passed across the gel to the membrane, transferring the proteins onto the membrane. The transferring method is also known as semi- dry method. The membrane is usually made of PVDF or nitrocellulose, both of which have advantages and disadvanatages that should be thoroughly researched prior to use.

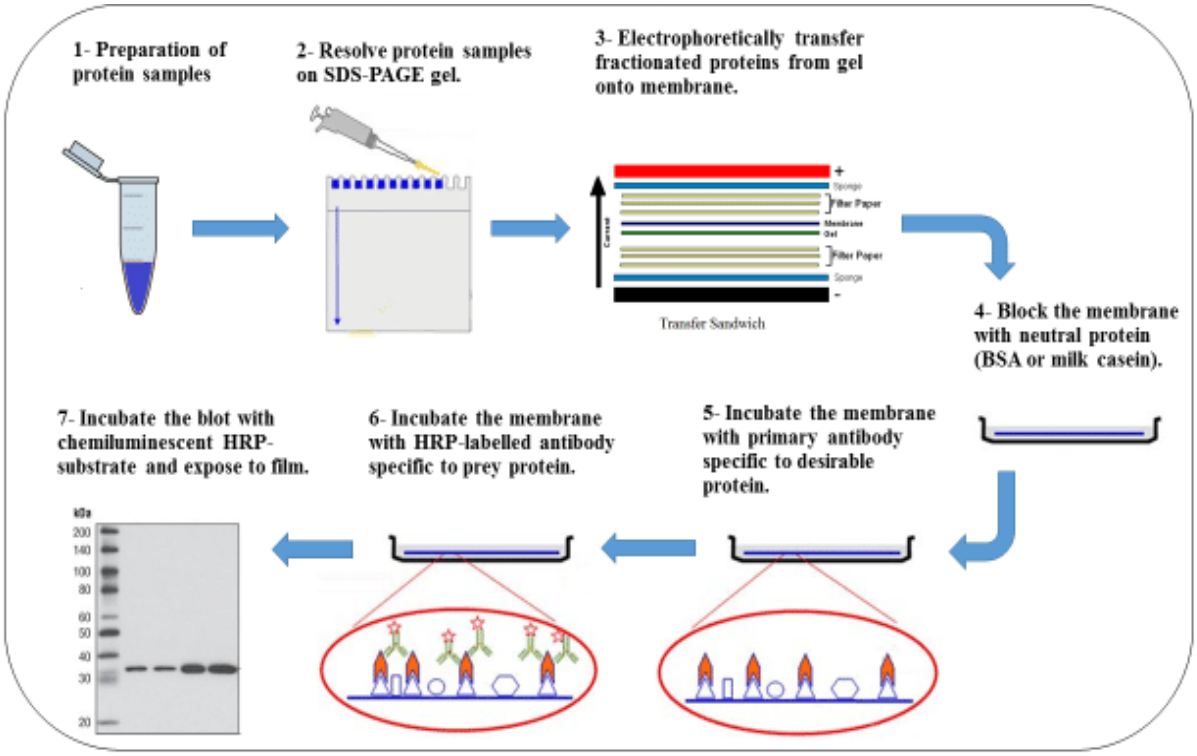
Step 4: Immunoblotting

The first step in immunoblotting is to wash the membrane and block it with non-specific protein. The non- specific protein binds to the surface of the membrane where protein is not already present. This will prevent antibody from binding to the membrane and giving a non- specific signal.Next, the primary antibody is added to the solution in which the membrane is floating. Remember that the primary antibody recognizes a specific amino-acid sequence of a particular protein. After a wash is conducted to remove unbound primary antibody, secondary antibody is added. Secondary antibody recognizes the primary antibody, and usually is conjugated with an enzyme, such as HRP (Horse Radish Peroxidase). Lastly, another wash is performed to remove unbound secondary antibody. Non-specific

binding of both the primary and secondary antibodies can occur, but thorough washing usually minimizes this problem. The amount of time the primary and secondary antibodies are applied, directly affects the specificity and strength of binding.

Step 5: Detection

The detection method used is dependent upon the enzyme to which the secondary antibody is conjugated. The most common enzyme used in Western Blotting is HRP, and the substrate used for detection is known as chemiluminescent substrate.Once the substrate has been added, the light being emitted can be detected with film or a photo imager.



4.DNA FINGER PRINTING

Introduction

- Every cell of our body contains DNA and approximately 99.9% of DNA is similar between two humans. Only 0.1% difference is responsible to make someone unique (except identical twins) and this 0.1% of DNA plays an important role in DNA Fingerprinting.

- As we know, only 3 % of our genome are coded and act for protein synthesis i.e. called gene and rest of 97% are non-coding, repetitive and junk; this junk DNA are utilized to perform DNA fingerprinting. The sequence structure and number of repeats varies between individuals and organisms. On this basis, the DNA print can be prepared.

- DNA fingerprinting technique was first developed by British Professor Sir Alec Jeffrey in 1984. He realized that we can detect variations in human DNA, in the form of mini satellites. Jeffrey created the first DNA profile using the restriction digestion length polymorphism. His method was actually a combination of RFLP and autoradiography.

Definition - "It is a molecular method to identify an individual or any living organism from their DNA sample by looking at unique patterns in their DNA."

Process of DNA Fingerprinting :-

1. Collection of biological samples.

2. DNA extraction.

3. Restriction digestion or PCR amplification.

4. Agarose gel electrophoresis, capillary electrophoresis or DNA sequencing.

5. Interpreting results.

1.Sample collection :-

DNA can be obtained from any biological samples.

Ex:any part of plant or animals.

2.DNA extraction :-

DNA can be extracted with different methods like CTAB DNA extraction methods, Proteinase K DNA extraction methods and Phenol-chloroform DNA extraction method or may be with any DNA extraction kit.

3.Restriction digestion or PCR amplifications :-

This process can be performed by three different techniques –

(i) RFLP analysis.

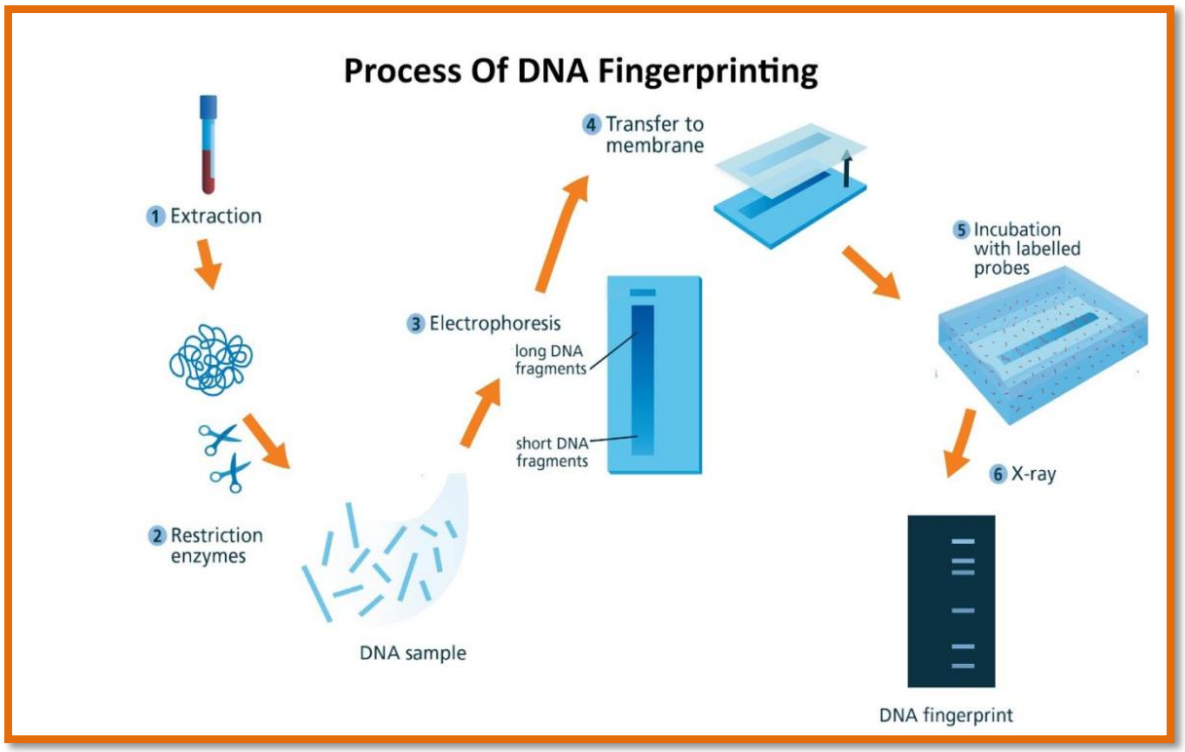
(ii) PCR analysis.

(iii)Real Time PCR analysis.

RFLP analysis :-

- RFLP is the first method used for DNA fingerprinting. It has been performed with restriction enzymes, which is used to cut the DNA. This resulted in thousands of pieces of DNA with a variety of different lengths.
- With the help of agarose gel electrophoresis these DNA has been separated on the basis of their size. Further the piece of DNA has been transferred to nylon membrane.

- The nylon membrane was incubated with radioactive probes (Probes are small fragments of mini satellite DNA tagged with radioactive phosphorous).
- The probes only attach to the complementary pieces of DNA and here, they attached to the mini satellites in the genome.
- The mini satellites attached with probes were then visualized by exposing the nylon membrane to X-ray film. When exposed to radioactivity a pattern of dark bands appeared on the film at the sight of the labelled DNA.
- This pattern is called the DNA fingerprint. To compare two or more different DNA fingerprints the different DNA samples were run side-by-side on the same electrophoresis gel.



5.SEPARATION,PURIFICATION OF BIOLOGICAL COMPOUNDS BY PAPER,THIN-LAYER AND COLUMN 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. Proteins can be purified based on characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase. Four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion. Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography.

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights . Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster .

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a “solid” phase or “a layer of a liquid adsorbed on the surface a solid support”.
- Mobile phase: This phase is always composed of “liquid” or a “gaseous component.”
- Separated molecules

The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses .

Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples.

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achieve a satisfactory separation within a suitable time interval.

Various chromatography methods have been developed to that end. Some of them include

- Column chromatography,
- Thin-layer chromatography (TLC),
- Paper chromatography,
- Gas chromatography,
- Ion exchange chromatography,
- Gel permeation chromatography,

- High-pressure liquid chromatography,
- Affinity chromatography .

1.Paper chromatography

In paper chromatography support material consists of a layer of cellulose highly saturated with water. In this method a thick filter paper comprised the support, and water drops settled in its pores made up the stationary "liquid phase." Mobile phase consists of an appropriate fluid placed in a developing tank. Paper chromatography is a "liquid-liquid" chromatography.

2.Thin-layer chromatography

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances used. in column chromatography (alumina, silica gel, cellulose) can be utilized. In this method, the mobile phase travels upward through the stationary phase.The solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent.

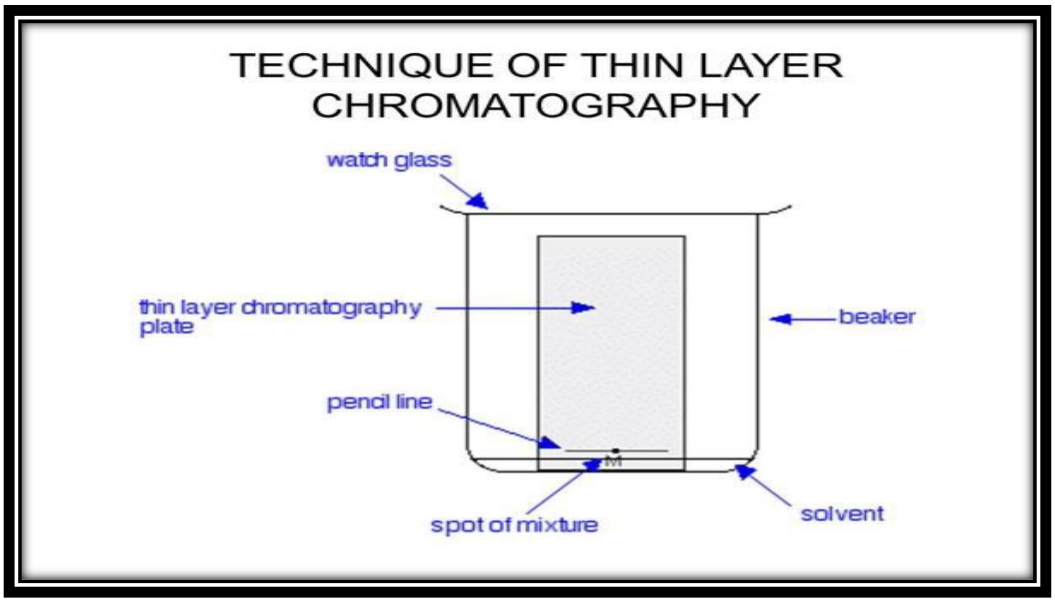
In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible coloured reactive product so as to identify their positions on the chromatogram. Formation of a visible colour can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the the distances travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol Rf. Rf. value is used for qualitative description of the molecules.

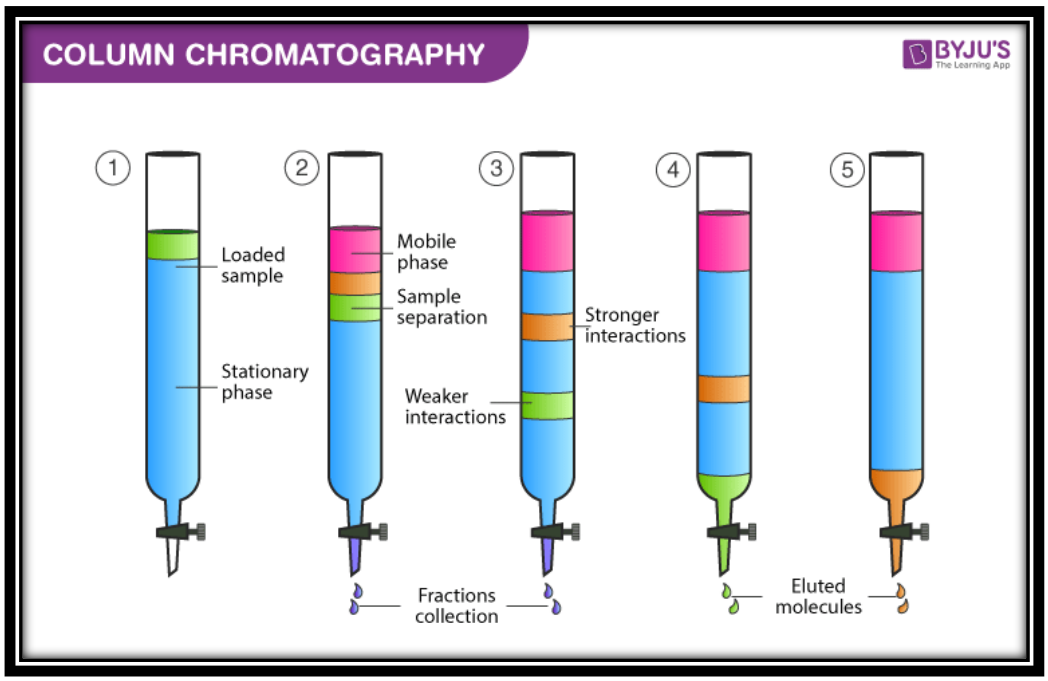
3**.Column chromatography**

Column chromatography is simple and the most popular separation and purification technique. Both solid and liquid samples can be separated and purified by column chromatography. Column chromatography consists of a stationary solid phase that adsorbs and separates the compounds passing through it with the help of a liquid mobile phase. On the basis of their chemical nature, compounds get adsorbed and elution is based on differential adsorption of a substance by the adsorbent.

Various stationary phases, such as silica, alumina, calcium phosphate, calcium carbonate, starch, and magnesia, and different solvent compositions based on the nature of compounds to be separated and isolated, are used in column chromatography. Optimization of the method is an important task in the separation of different groups of compounds in extracts. In column chromatography, a cylindrical glass tube, which is plugged at the bottom by a piece of glass wool or porous disc, is filled with slurry (adsorbent) and a suitable solvent.

Samples to be separated are mixed with silica and introduced at the top of the column and allowed to move with the solvent. With polarity differences, compounds are adsorbed at different regions and desorbed with suitable solvent polarity. The compound of higher adsorption ability will be adsorbed at the top and that with the lower one will be at the bottom. By adding the solvent at the top, compounds get desorbed and pass through the column and this process is called elution.


TECHNIQUE OF THIN LAYER CHROMATOGRAPHY
watch glass
thin layer chromatography plate
beaker
pencil line
spot of mixture
solvent


COLUMN CHROMATOGRAPHY
BYJU'S
1
2
3
4
5
Loaded sample
Stationary phase
Mobile phase
Sample separation
Weaker interactions
Stronger interactions
Fractions collection
Eluted molecules

6.CLEANING AND STERILIZATION OF GLASS AND PLASTIC WARE FOR CELL CULTURE

Good laboratory technique demands clean glassware because the most carefully executed piece of work may give an erroneous result if dirty glassware is used. In all instances, glassware must be physically and chemically clean and in many cases, it must be bacteriologically clean or sterile. All glassware must be absolutely grease-free. The safest criterion of cleanliness is uniform wetting of the surface by distilled water. This is especially important in glassware used for measuring the volume of liquids. Grease and other contaminating materials will prevent the glass from becoming uniformly wetted. This in turn will alter the volume of residue adhering to the walls of the glass container and thus affect the volume of liquid delivered. Furthermore, in pipettes and burettes, the meniscus will be distorted and the correct adjustments cannot be made. The presence of small amounts of impurities may also alter the meniscus.

CLEANING LABORATORY GLASSWARE

- Wash lab ware as quickly as possible after use. If lab ware is not cleaned immediately, it may become impossible to remove any residue.
- If a thorough cleaning is not possible immediately, soak glassware in water.
- Most new glassware items are slightly alkaline in reaction. For precision chemical testing, new glassware should be soaked for several hours in acid water (a 1% solution of hydrochloric or nitric acid) before proceeding with a regular washing proceedure.
- Brushes with wooden or plastic handles are recommended as they will not scratch or otherwise abrade the glassware's surface

GLASSWARE CLEANERS

 When washing, soap, detergent, or cleaning powder (with or without an abrasive) may be used. Cleaners for glassware include Alconox, Dural, M&H, Lux, Tide, and Fab.

 The water should be hot for glassware that is exceptionally dirty; a cleaning powder with a mild abrasive action will give more satisfactory results. The abrasive should not scratch the glass.

 During the washing, all parts of the glassware should be thoroughly scrubbed with a brush. This means that a full set of brushes must be at hand, including brushes to fit large and small test tubes, burettes, funnels, graduates, and various sizes of flasks and bottles.

 Do not use cleaning brushes that are so worn that the spine hits the glass. Serious scratches may result. Scratched glass is more prone to break during experiments.

 Do not allow acid to come into contact with a piece of glassware before the detergent (or soap) is thoroughly removed. If this happens, a film of grease may be formed.

RINSING

- It is imperative that all soap, detergents, and other cleaning fluids be removed from glassware before use. This is especially important with the detergents, slight traces of which will interfere with serologic and cultural reactions.

- After cleaning, rinse the glassware with running tap water. When test tubes, graduates, flasks, and similar containers are rinsed with tap water, allow the water to run into and over them for a short time, then partly fill each piece with water, thoroughly shake and empty at least six times. Pipettes and burettes are best rinsed by attaching a piece of rubber tubing to the faucet and then attaching the delivery end of the pipettes or burettes to a hose, allowing the water to run through them. If the tap water is very hard, it is best to run it through a deionizer before using.

• Rinse the glassware in a large bath of distilled water. Rinse with distilled water. To conserve distilled water, use a five gallon bottle as a reservoir. Store it on a shelf near your clean-up area. Attach a siphon to it and use it for replenishing the reservoir with used distilled water. For sensitive microbiologic assays, meticulous cleaning must be followed by rinsing 12 times in distilled water.

STERILIZING CONTAMINATED GLASSWARE

 Glassware which is contaminated with blood clots, such as serology tubes, culture media, petri dishes, etc., must be sterilized before cleaning. It can best be processed in the laboratory by placing it in a large bucket or boiler filled with water, to which 1-2% soft soap or detergent has been added, and boiling for 30 minutes. The glassware can then be rinsed in tap water, scrubbed with detergent, and rinsed again.

 You may autoclave glassware or sterilize it in large steam ovens or similar apparatus. If viruses or spore-bearing bacteria are present, autoclaving is absolutely necessary.

HANDLING AND STORING

• To prevent breakage when rinsing or washing pipettes, cylinders, or burettes, be careful not to let tips hit the sink or the water tap.

• Dry test tubes, culture tubes, flasks, and other lab ware by hanging them on wooden pegs, placing them in baskets with their mouths downward and allowing them to dry in the air, or placing them in baskets to dry in an oven. Drying temperatures should not exceed 140 °C. Line the drying basket with a clean cloth to keep the vessel mouths clean.

• Dry burettes, pipettes, and cylinders by standing them on a folded towel. Protect clean glassware from dust. This is done best by plugging with cotton,

corking, taping a heavy piece of paper over the mouth, or placing the glassware in a dust-free cabinet.

- Store glassware in specially designed racks. Avoid breakage by keeping pieces separated.

- Do not store alkaline liquids in volumetric flasks or burettes.Stoppers or stopcocks may stick.

7.PREPARATION OF MEDIA

AIM : To prepare desired medium for the given Animal cell culture.

PRINCIPLE:

All the Animal cells can be grown in a liquid culture medium consisting of a mixture of vitamins, salts, glucose, amino acids and growth factors. Moreover, Calf serum is an easily available source of growth and attachment factors. Antibiotics are added to prevent the growth of bacteria. Under these conditions cells will grow at physiological pH (7.4) and at body temperature (37ºC) to form a monolayer on the culture vessels.

MATERIALS REQUIRED-

- Medium Adult bovine serum Membrane filter (Millipore 0.45µ)
- Sterilize Double distilled water 1000 ml
- 1 litre measuring cylinder
- 100 ml measuring cylinder
- 1 litre filtration flask
- Medium storage bottles.

METHOD-

Sterilize the laminar air flow by UV irradiation for 45 minutes before using it.

1. Take 500ml of sterile double distilled water in a 1000 ml measuring cylinder.
2. Transfer the contents of the powdered medium into 1 litre measuring cylinder add 3.7 gms of $NaHCO_3$ in the absence of CO2 incubator.
3. Mix thoroughly to dissolve the powdered medium, and add penicillin /streptomycin/gentamicin.
4. Fill the cylinder with1 litre double distilled water mix and transfer to sterile 2 litre flask and mix. Pinkish red colour of the medium indicates normal pH range.

5. Assemble the filter sterilization set-up and carry out the filtration under negative pressure.
6. Prepare 400 ml of medium containing 10% Adult bovine serum using 100 ml measuring cylinder and store in a 500 ml sera lab bottle.
7. Transfer the remaining medium without serum into big glass bottles.
8. Store the medium in refrigerator, dispose the used membrane and immerse the used glassware in water for washing.
9. Different types of medium is used for various kind of Experiments.

Hank's balanced salt solution

S.No	Constituents	Amount (gm/lt)
1	Nacl	8
2	Kcl	0.4
3	$Cacl_2$	0.14
4	$Mgso_4.7H_2o$	0.2
5	$MgCl_2.6H_2o$	0.1
6	$Na_2HPo_4.H_2o$	0.09
7	$KH2Po_4$	0.06
8	$NaHCo_3$	0.35
9	D.Glucose	1
10	Phenyl red	0.01gm
11	Gas phase (CO_2)	5%

Earle's Balanced Salt Solution

S.No	Constituents	Amount (gm/lt)
1	NaCl	0.68
2	KCl	0.4
3	$CaCl_2$	0.02
4	NH $NaH_2Po_4.H_20$	0.14
5	$MgSo_4.H_20$	0.2
6	$NaHCo_3$	0.22
7	D.Glucose	1
8	Phenyl red	0.01
9	Gas Phase($C0_2$)	5%