BSc ZOOLOGY – V SEMESTER

IMMUNOLOGY AND BIOTECHNOLOGY

PRACTICAL MANUAL

I. Immunology

1. DEMONSTRATION OF AGGLUTIATION –ABO BLOOD GROUPING USING KIT

Aim

To found out my blood group by agglutination test.

Materials required

Glass slide, Sterilized needle, Antiserum „A" and Antiserum „B'.

Principle involved

In the human blood, two types of substances are found namely antigens and antibodies. The antigens are present in RBC. Antibodies are present in plasma.Landsteiner (1900) classified four different blood group in human beings according to the type of antigens present or antigens absent. They are A, B, AB and O groups. The blood group has antigen A and antibody B. The blood group B has antigen B and antibody A.
The AB blood group has both the antigens (A and B), but no antibodies. The blood group O has no antigen, but with both antibodies (A and B). Agglutination (clumping) takes place between the corresponding antigens and antibodies. For e.g. clumping takes place between antigen A and antibody B. Similarlyclumping takes place between antigen B and antibodies A. Based on this agglutination the blood group is referred as A and B group. Agglutination takes place between both antigen and antibodies, the blood group is AB, if agglutination does not take place, it is O blood group. Thus the blood group is determined by based on the principle.

Procedure

My left hand ring finger was sterilized and pricked with sterilized needle. A drop of blood was placed at one end of the glass slide and another drop at the other end. The antiserum A and B was added to the blood drops separately and stirred. Then the blood group was identified by observing the agglutination.

Result

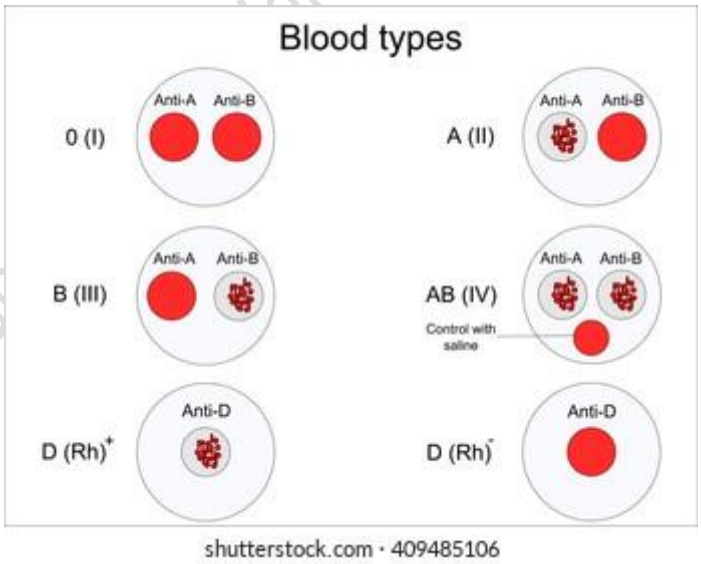
According to the agglutination test (Fig.). my blood group was identified as.....group because there was..... Agglutination with.....antiserum.

Significance of blood group

The grouping of blood is of great importance in blood transfusion if unmatched blood is transfused to the patient he/she will die due to agglutination. So the blood of the donor and recipient are tested before transfusion.

1. Thus AB group person can receive blood from all the 'four groups, because it does not have any antibody to agglutinate. Therefore AB group persons are called „universal recipients".
2. Group „O" person cannot receive blood from any group except „O" group, because „O" group blood has both antibodies A and B for agglutination. At the same time since both antigens are absent.
This group person is called "universal donor".
3. Group A person can receive blood from A group and O group and not from B and AB group.
4. Group B person can receive blood from B group and O group and not from A and AB group.

Agglutination test for blood grouping



2. DEMONSTRATION OF PRECIPITATION (VDRL TEST) USING KIT

Introduction and Aim:

The venereal disease research laboratory (VDRL) test is a nonspecific microflocculation test for the screening of syphilis. It uses antigen-containing cardiolipin, lecithin, and cholesterol and measures both **IgG** and **IgM antibodies** to lipoidal and lipoproteins released as a result of damage to host cells and also to cardiolipin released from treponemes. VDRL test is easy to perform and inexpensive, so it is commonly used in the screening of the population for syphilis. Without some other evidence for the diagnosis of syphilis, a reactive nontreponemal test does not confirm *Treponema pallidum* infection.

Sample:

1. Serum (plasma can not be used)
2. Cerebrospinal fluid (CSF)

Acceptable CSF and serum specimens should not contain particulate matter that would interfere with reading test results. Serum samples that are excessively hemolyzed, contaminated, and turbid are not suitable for testing.

Principle of VDRL Test

Non-treponemal antigen (cardiolipin-cholesterol-lecithin) is used to detect the presence of "reagin antibodies" (IgM and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material, and possibly cardiolipin released from the treponemes) in the patient's serum.

When the heat-inactivated serum (to destroy **complement**) of a patient reacts with freshly prepared non-treponemal antigen, a flocculation reaction (Ag-Ab complex are suspended) occurs. The flocculation can be observed by using a microscope with a 10x objective and 10 x eyepieces.

Reactive VDRL test serum can be quantitated to obtain the titre of "reagin antibodies" by using the serial double dilution method.

Result and Interpretation of VDRL test

- Reactive: medium or large clumps
- Weakly reactive: small clumps
- Nonreactive: no clumping or very slight roughness

- Report titers in terms of the highest dilution that produces a reactive (not weakly reactive) result

VDRL test is positive in most cases of primary syphilis and is almost always positive in secondary syphilis. The titer of reagin antibodies decreases with effective treatment, so the VDRL test can be used to determine the treatment response of syphilis.

Limitation of the Test

A. False positive VDRL test result

1. Reagin antibodies may be produced in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs such as:
    - Leprosy
    - Hepatitis B
    - Infectious Mononucleosis
    - Various autoimmune diseases
2. VDRL may be reactive in persons from areas where yaws are endemic. As a rule, residual titers from these infections will be <1:8.
3. Nontreponemal test titers of persons treated in latent or late stages of syphilis or who have become reinfected do not decrease as rapidly as do those from persons in the early stages of their first infection. In fact, these persons may remain "**serofast,"** retaining a low -level reactive titer for life.

B. False negative VDRL test

It can be seen because of the prozone phenomenon (no flocculation due to antibody excess). In that case test serum has to be diluted further to obtain a zone of equivalence (where maximum flocculation of Ag-Ab occurs).

3.RADIAL IMMUNODIFFUSION USING KIT

Aim:

To determine the concentrations of antigen by radial immuno diffusion method.

Principle

In radial immuno diffusion method the antiserum is incorporated in the agar and the antigen is placed in wells. The antigen diffuses radially from the well into the agar containing antiserum. The antigen reacts with the antiserum, a ring shaped precipitin band is formed around the well. The concentration of the antigen can be calculated by measuring the diameter of the precipitin ring.
The radial immuno diffusion the antigen alone in the agar plate hence it is called single radial immuno diffusion.

Materials required

Borate buffer, agar, 1% BSA antiserum, micro slide, cork borer, petridish and cotton.

Procedure

Preparation of agar: 1g of agar was dissolved in 100 ml of Borate saline solution (5 ml of Borate buffer and 95 ml of physiological saline form the Borate saline solution). In a conical flask, by boiling in a water bath, 2.5 ml of hot agar solution was transferred to a micro slide with the help of a pipette. The agar solution was poured to a depth of 2 to 15 minutes. The agar coated slide was called agar plate. After cooling 1% BSA antiserum was added on the agar plate. Antiserum diffused uniformly in the agar plate kept in the refrigerator for 30 minutes. With help of a cork borer, a well was cut on the agar plate or in the centre of the slide. Antigen diffused out radially when it was diffused out. It came in contact with the antibody to form a precipitin ring.

Results

The diameter of the ring (Fig. 192) was a measure of the concentration of the antigen. The diameter of the ring depends on the concentration of antigen. The log of the diameter of the ring is proportional to the antigen concentration. The higher the antigens content the longer the ring.

Inference : The diameter of the ring is a measure of the concentration of the antigen. The diameter of the ring depends on the concentration of antigen. The log of the diameter of the ring is proportional to the antigen concentration The higher the antigen content, thelarger the ring.

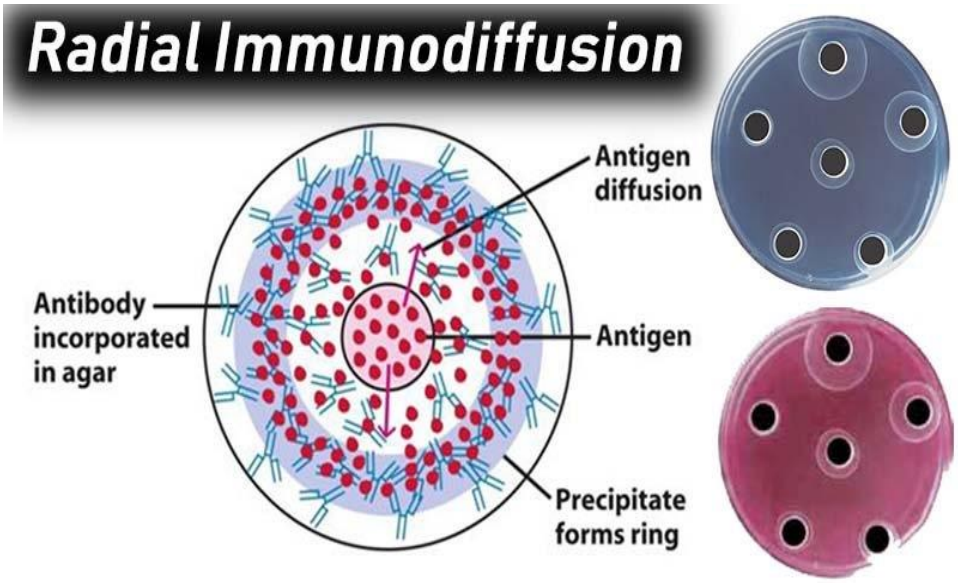


Fig. Radial immunodiffusion ring.

4. HISTOLOGY OF LYMPHOID ORGANS

A. SPLEEN

Location and Structure of Spleen:

Spleen lies in between the fundus of the stomach and the diaphragm. The spleen is soft, highly vascular and dark purple in colour. The spleen is the largest single mass of lymphatic tissue in the body. Its average weight in the adult is about 150 gm. Its long axis lies in the line of the tenth rib. The spleen has a long fissure, the hilium, near its lower portion. Except at the hilum, the surface of the spleen is covered by a layer of visceral peritoneum (= serous coat). Next to the visceral peritoneum, there is a capsule. The trabeculae arise from the capsule that extend into the substance of the spleen.

White Pulp:

The lymphoid tissue (mostly lymphocytes) surround the arterioles, forming masses or nodules, the splenic nodules (= Malpighian bodies) which appear whitish and hence called the white pulp of the spleen.

Red Pulp:

The remaining part of the splenic tissue appears reddish due to red blood corpuscles and hence, called the red pulp of spleen. It contains numerous venous sinusoids which are large and complex cavities containing blood. The venous sinusoids are separated by areas of tissue rich in macrophages attached to the reticulum of the spleen called Splenic cords of Billroth.

The term ‘cord’ is perhaps misleading, because these areas of perivascular tissue form a continuous network throughout the spleen and have numerous cavities between the cells through which blood can pass. In some mammals like mouse and cat, there are no sinusoids and the majority of the red pulp is composed of splenic cord tissue.

Blood circulation in spleen:

Splenic artery → Arterioles → Venous sinusoids → Venules → Splenic vein.

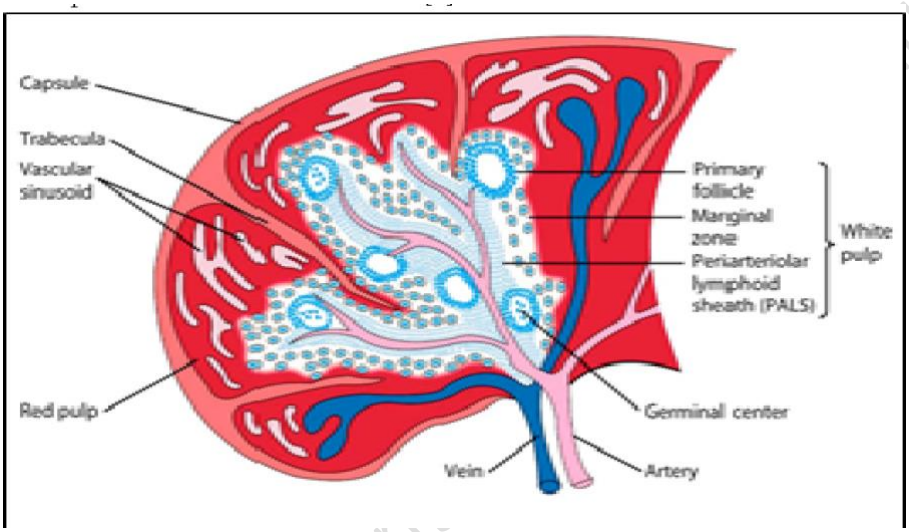
Functions of Spleen:

(1) Phagocytosis:

(2) Haemopoiesis:

(3) Immune Response:

(4) Storage of Erythrocytes:



B.THYMUS

Thymus is a greyish, flat, bilobed lymphoid organ situated above the heart and extending into the neck on the front and sites of trachea. It develops from the epithelium of third and fourth pharyngeal pouches and, on maturity, acts as the site of development and maturation of lymphocytes named thymus-derived lymphocytes or T-lymphocytes or T-cells.

Each lobe of thymus is surrounded by a capsule and is divided into a series of lobules, which are separated from each other by strands of connective tissue called trabeculae. Each lobule is organized into two compartments-outer and inner. The outer component is called cortex, whereas the inner component is called medulla

The cortex is densely packed with thymocytes, whereas the medulla is sparsely populated with thymocytes. Thymocytes develop from prothymocytes. The latter are produced in bone marrow, migrate through blood stream, enter the cortex of the thymus, and act as thymocytes. Thymocytes divide rapidly in the cortex and give rise to T-lymphocytes.

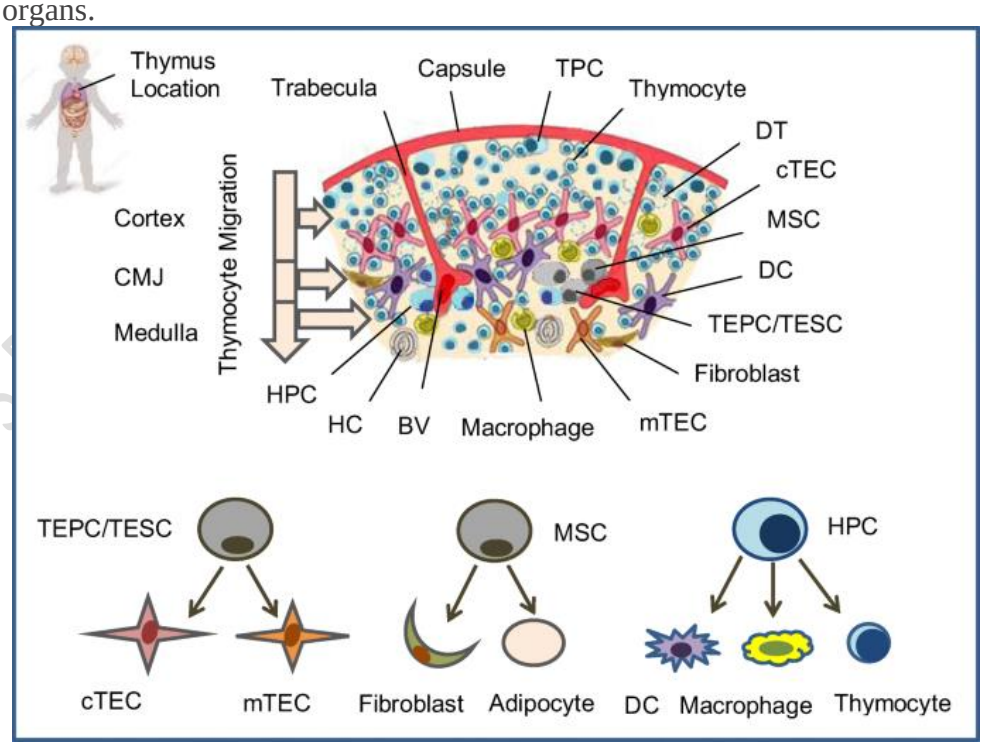
Of the T-lymphocytes produced in thymus only 5% leave the thymus as viable cells. Though the reason for this apparent wasteful process is not known, some believe that it is the elimination of T-lymphocyte clones that react against self.

Both the cortex and the medulla of the thymus are criss-crossed by a three dimensional network consisting of epithelial cells, dendritic cells, and macrophages, which make up the framework of the organ and contribute to the growth and maturation, of thymocytes.

Some epithelial cells of the outer cortex possess long membrane extensions that surround as many as 50 thymocytes. These cells are called nurse cells. Other epithelial cells of the cortex have long interconnecting cytoplasmic extensions that form a network and have been found to interact with many of the thymocytes when they traverse the cortex.

The function of the thymus is to generate T-lymphocytes and to confer immunological competence on to them during their stay in the organ. T-lymphocytes so educated in the thymus become capable of mounting cell-mediated immune response against appropriate antigen.

This is effected under the influence of the thymic microenvironment and several hormones such as thymosin and thymopietin produced by the epithelial cells of the thymus. The competent T-lymphocytes immediately move from thymus to the secondary or peripheral lymphoid organs.



C. LYMPHNODE:

Lymph nodes are small, encapsulated, bean-shaped structures clustered at junctions of the lymphatic vessels which are distributed throughout the body. Lymph nodes contain a reticular network packed with lymphocytes, macrophages and dendritic cells, and filter out pathogenic microorganisms and antigens from the lymph. As the lymph percolates through a lymph node, any pathogen or antigen that is brought in with the lymph is trapped by the phagocytic cells and dendritic cells.

A lymph node consists of three regions: the cortex, the paracortex, and the medulla.Cortex is the outermost region and contains several rounded aggregates of lymphocytes (mostly B-lymphocytes), macrophages, and follicular dendritic cells arranged in primary follicles. Each follicle has a pale-staining germinal centre surrounded by small dark-staining lymphocytes.

The deeper region lying beneath the cortex is the paracortex. It is the zone between the cortex and the medulla. Paracortex possesses large number of T-lymphocytes and also contains inter-digitating dendritic cells thought to have migrated from tissues to the lymph node.

Because of the presence of large number of T-lymphocytes in it. the Para-cortex is also referred to as a thymus-dependent area in contrast to the cortex which is a thymus-independent area. Medulla, the inner most region of lymph node, is more sparsely populated with lymphoid-lineage cells. Of the lymphoid-lineage cells present, many are plasma cells actively secreting antibody molecules.

Each lymph node has a number of lymph vessels called afferent lymphatic vessels, which pierce the capsule of a lymph node at numerous sites and empty lymph into the sub-capsular sinus. The lymph now percolates slowly inward through the cortex, paracortex, and medulla, allowing phagocytic cells and dendritic cells to trap pathogens and antigens carried by the lymph.

The lymph then is drained into a single large lymph vessels called efferent lymphatic vessel that carries the lymph to the thoracic duct, which empties into a large vein in the neck.

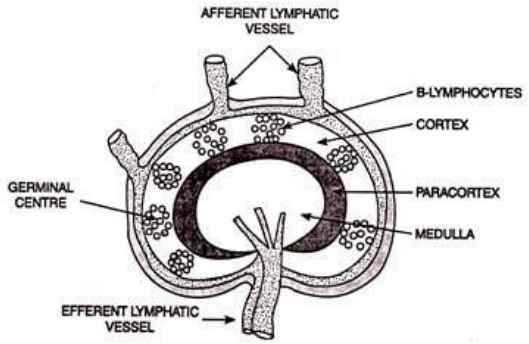


FIG. 42.3. Lymph node structure showing cortex, paracortex, medulla, afferent and efferent lymphatic vessels, and lymphocytes.

D. BONE MARROW

Bone marrow is the site of origin and development of B-lymphocytes or B-cells (bone marrow derived lymphocytes) in mammals particularly in humans and mice after birth. Before birth, the yolk sac, foetal lever, and total bone marrow are the major sites of B-lymphocyte maturation. Bone marrow, therefore, is the mammalian equivalent of the bursa of Fabricius in birds.

Development of B-lymphocytes (B-cells) begins with the differentiation of lymphoid stem cells into the earliest distinctive progenitor B cells (pro-B cell), which proliferate within the bone marrow filling the extravascular spaces between large sinusoids in the shaft of a bone.

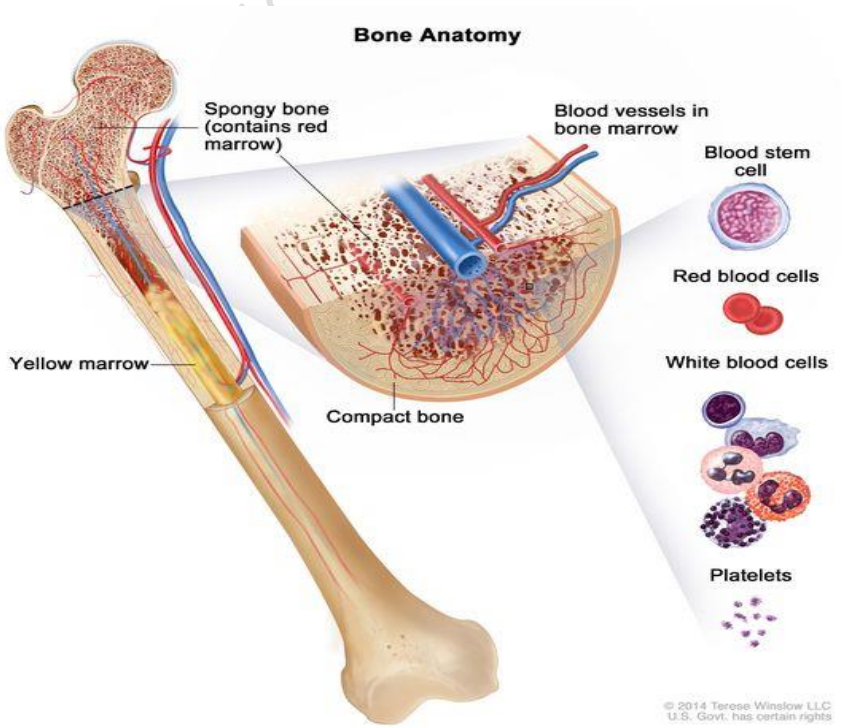
Proliferation and differentiation of pro-B cells into precursor B cells (pre-B cells) requires the microenvironment provided by the bone marrow stromal cells.

The stromal cells within the bone marrow:

(1) Interact directly with the pro-B and pre-B cells and

(2) Secrete various cytokines that are required for development.

Bone marrow is not the site of origin and development of B-lymphocytes (B-cells) in all mammals. In cattle and sheep, the fietal spleen is the primary lymphoid tissue wherein the maturation, proliferation, and diversification of B-cells take place during early gestation.



II. ANIMAL BIOTECHNOLOGY

1. STUDY OF THE FOLLOWING TECHNIQUES THROUGH PHOTOGRAPHS/ VIRTUAL LAB.

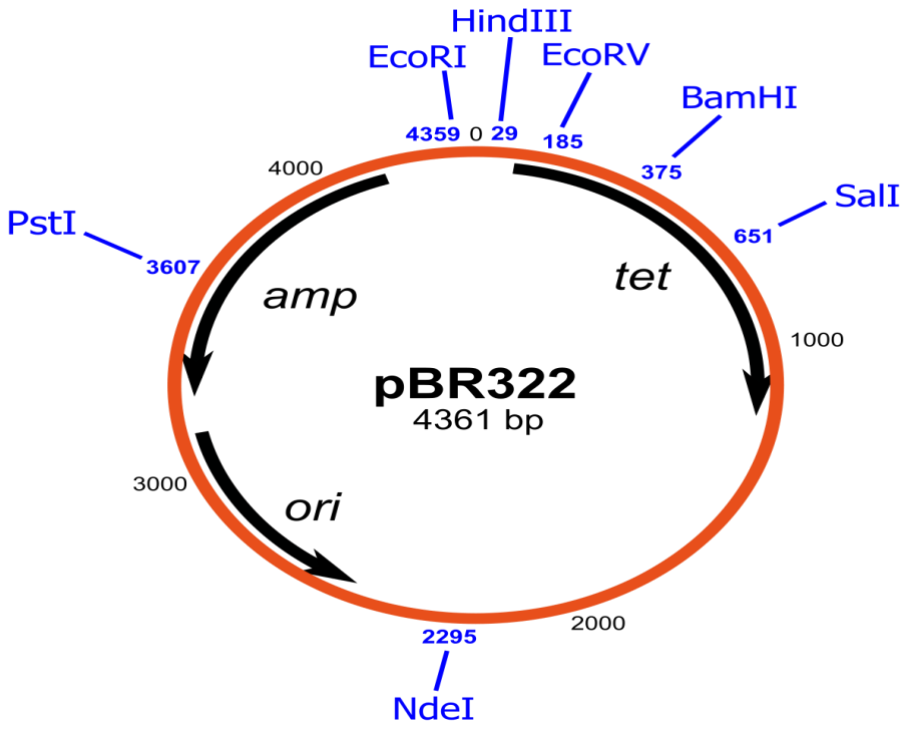
a. Identification of vectors.

i. PLASMID

Plasmids are the extra-chromosomal, self-replicating, and double stranded closed and circular DNA molecules present in the bacterial cell. A number of properties are specified by plasmids such as antibiotic and heavy metal resistance, nitrogen fixation, pollutant degradation, bacteriocin and toxin production, colicin factors, etc.

Plasmids have following advantages as cloning vehicle (Cohen et a. 1973):

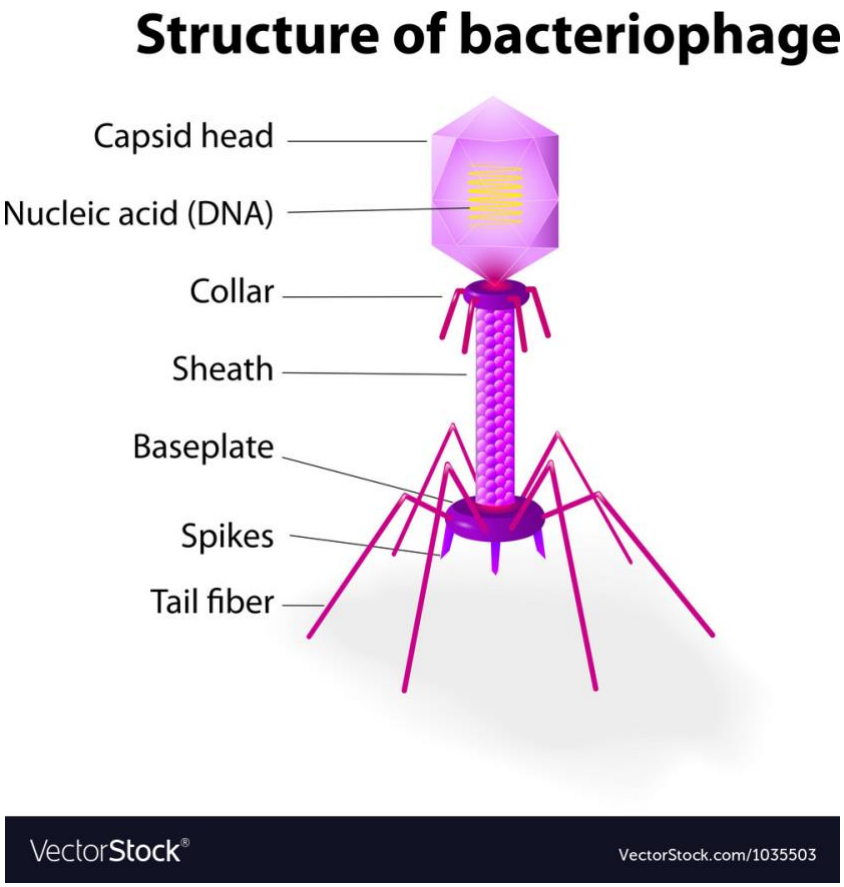
1. It can be readily isolated from the cells.
2.It possesses a single restriction site for one or more restriction enzymes.
3. Insertion of foreign DNA does not alter the replication properties.
4. It can be reintroduced into cell.
5. Selective marker is present.
6. Transformants can be selected easily by using selective medium.



(ii) BACTERIOPHASE

The bacteriophage has linear DNA molecule, a single break will generate two fragments, foreign DNA can be inserted to generate chimeric phage particle. But as the capacity of phage head is limited, some segments of phage DNA, not having essential genes, may be removed. This technique has been followed in λ (Lambda) phage vectors to clone large foreign particle.

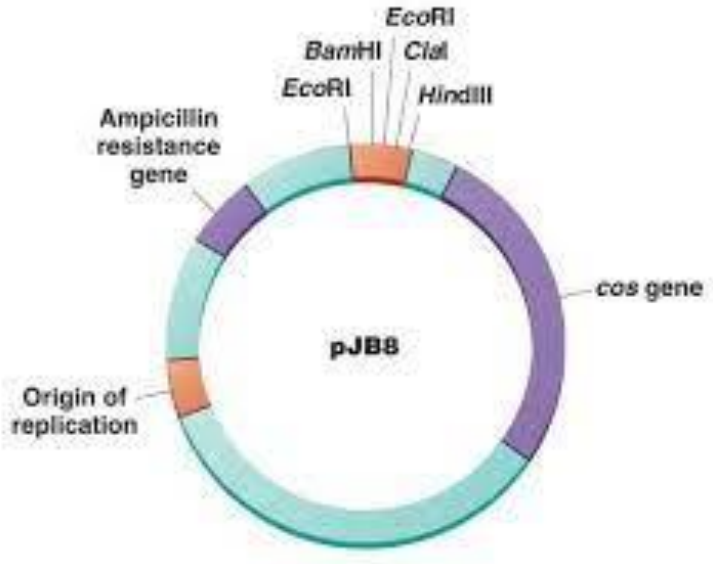
Plasmid can clone up to 20 to 25 kb long fragments of eukaryotic genome. The examples of different Lambda phage vectors are λ gt 10, λ gt 11, EMBL 3, etc. M-13 is a filamentous bacteriophage of E. coli whose single stranded circular DNA has been modified variously to give rise M-13 series of cloning vectors.



(iii) cosmids

1. A cosmid, first described by Collins and Hohn in 1978, is a type of hybrid plasmid with a bacterial “ori” sequence and a “cos” sequences derived from the lambda phage.
2. Cosmid is a circular ds DNA
3. . It has two complementary single-stranded regions at both ends of a plasmid DNA. The two cos-ends form a duplex by base pairing.
4. . The cosmid DNA does not code for phage proteins and host cell lysis. 4. It does not involve in ,multiplication of phage particles.
5. . It has an origin of replication from plasmid DNA for independent replication.
6. . It has selectable marker genes and gene cloning sites of plasmid DNA
7. . The cosmid DNA is packed within protein coat of bacteriophage to form inactive phage particles. Cos-site is a prerequsites for invitro packaging of cosmid in phage protein coat.
8. . After infection, the cosmid DNA does not integrate into host chromosomal DNA. It exits as a definite extra chromosomal DNA and replicates independently.

Examples: Col EI cosmid, pHC 79, pJB8, pWE cosmid, etc.



b. TRANSGENIC ANIMALS

(i) Transgenic Mice:

1. Animal cells, like the protoplasts of plant cells, can take up foreign chromosomes or DNA directly from the environment with a very low efficiency (in the presence of calcium phosphate). Directly injecting the DNA greatly improves the efficiency.
2. For example, transgenic mice are now routinely prepared by injecting DNA either into oocytes or one or two- celled embryos obtained from female mice after appropriate hormonal treatment.
3. After injection of about 2 picoliters (2×10^{-12} liters) of cloned DNA, the cells are re implanted into the uteruses of receptive female hosts. In about 15% of these injections, the foreign DNA incorporates into the embryo.
4. Transgenic animals are used to study the expression and control of foreign eukaryotic genes. In 1988, a transgenic mouse prone to cancer was first genetically engineered animal to be patented. Thus mouse provides an excellent model for studying cancer. (A controversy arose as to whether engineered higher organisms should be patentable; currently they are).
5.Mice have already been successfully transfected with a rat growth-hormone gene.

(ii) TRANSGENIC FISH

1. In 2015, salmon became the first genetically engineered organism approved for consumption in the United States.
2. To produce this “AquAdvantage” salmon, researchers at the company AquaBounty introduced a growth hormone gene that resulted in faster growth of the salmon, reducing their market time from 3 years to 18 months.
3. Raising these transgenic salmon requires fewer resources than normal salmon, thus contributing to the production of more sustainable food products.
4. The larger salmon in the background is a sister to the smaller one in the foreground. The only difference is that a gene for faster growth is present in the larger fish. Both salmon reach the same size at maturity, but the smaller fish will take about twice as long to reach the mature size.

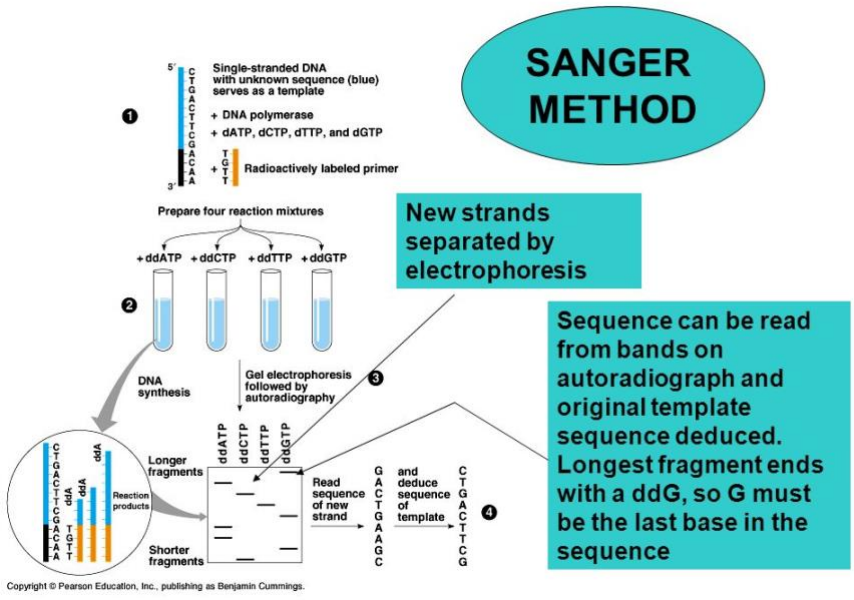
(iii) TRANSGENIC COW

1.Polling, the process of removing the horns of cattle, is a painful process for the animals, but is essential to avoid injuries while packing them into trucks for transportation.
2. Alison Van Eenennaam at the University of California, Davis proposes a solution using genome engineering to produce elite cow breeds without horns. Her idea involves introducing the gene responsible for the hornless state of Aberdeen Angus cows into Holstein cows to eliminate their horns.
3. Van Eenennaam is also currently working on a "Boys Only" project, wherein she intends to use CRISPR to introduce a gene that triggers all male characteristics in cows. The resulting animals will possess higher muscle content, potentially aiding sustainable and economical beef production in the future.

C.DNA SEQUENCING (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 T4 with different limiting triphosphates. 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.

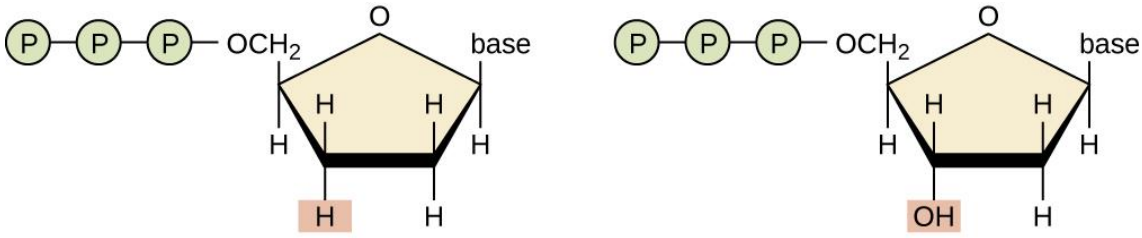
- Sanger's method of gene sequencing is also known as dideoxy chain termination method. It generates nested set of labelled fragments from a template strand of DNA to be sequenced by replicating that template strand and interrupting the replication process at one of the four bases.
- Four different reaction mixtures are produced that terminates in A. T. G or C respectively.



Principle

- A DNA primer is attached by hybridization to the template strand and deoxynucleosides triphosphates (dNTPPs) are sequentially added to the primer strand by DNA polymerase.
- The primer is designed for the known sequences at 3' end of the template strand.
- M13 sequences is generally attached to 3' end and the primer of this M13 is made.

- The reaction mixture also contains dideoxynucleoside triphosphate (ddNTPs) along with usual dNTPs.
- If during replication ddNTPs is incorporated instead of usual dNTPs in thegrowing DNA strand then the replication stops at that nucleotide.
- The ddNTPs are analogue of dNTPs
- ddNTPs lacks hydroxyl group (-OH) at c3 of ribose sugar, so it cannot make phosphodiester bond with nest nucleotide, thus terminates the nucleotide chain
- Respective ddNTPs of dNTPs terminates chain at their respective site. For example ddATP terminates at A site. Similarly ddCTP, ddGTP and ddTTP terminates at C, G and T site respectively.

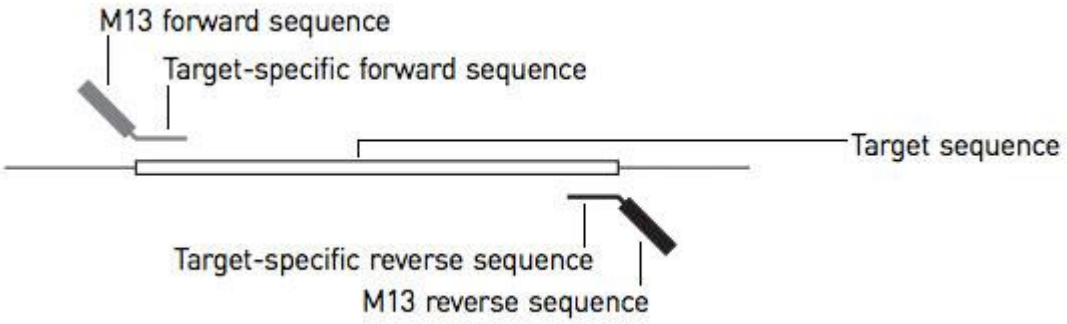


dideoxynucleotide (ddNTP) deoxynucleotide (dNTP)

Procedure

1. Template preparation:

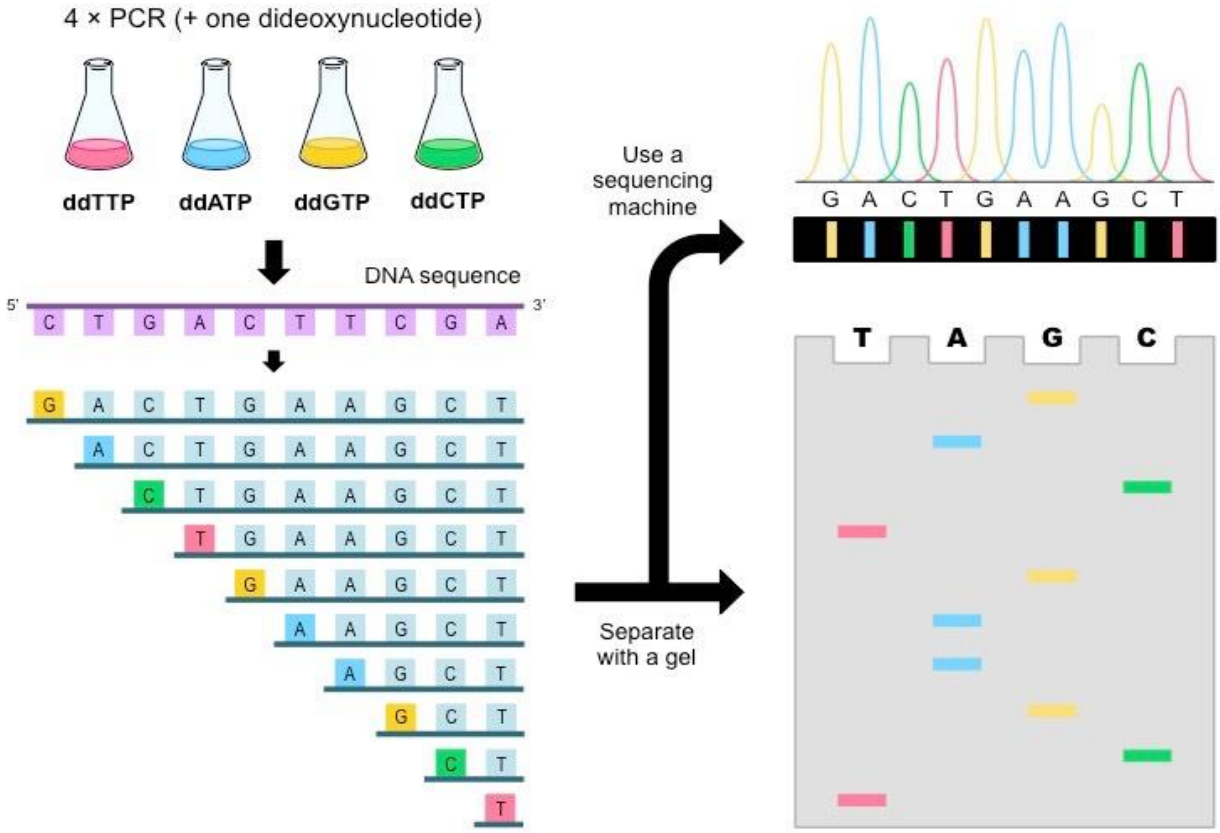
- Copies of template strand to be sequenced must be prepared with short known sequences at 3' end of the template strand.
- A DNA primere is essential to initiate replication of template , so primer preparation of known sequences at 3'end is always required.
- For this purpose a single stranded cloning vector M13 is flanked with template strand at 3'end which serves as binding site for primer.



2. Generation of nested set of labelled fragments:

- Copies of each template is divided into four batches and each batch is used for different replication reaction.
- Copies of standard primer and DNA polymerase I are used in all four batches.

- To synthesize fragments that terminates at A, ddATP is added to the reaction mixture on batch I along with dATP, dTTP,dCTP and dGTP, standard primer and DNA polymerase I.
- Similarly, to generate, all fragments that terminates at C, G and T, the respective ddNTPs ie ddCTP,ddGTP and ddTTP are added respectively to different reaction mixture on different batch along with usual dNTPs.



3. Electrophoresis and gel reading:

- The reaction mixture from four batches are loaded into four different well on polyacrylamide gel and electrophoresed.
- The autoradiogramof the gel is read to determine the order of bases of complementary strand to that of template strand.
- The band of shortest fragments are at the bottom of autoradiogram so that the sequences of complementary strand is read from bottom to top.

D. DNA FINGER PRINTING

This is also known as ‘DNA PROFILING’ o ‘DNA TYPING’. DNA fingerprinting is a technique to identify a person on the basis of his/her DNA specificity.

The practice of using thumbs impression of a person, as an identifying mark is very well known since long.

The study of finger, palm and sole prints is called dermatoglyphics and it has been a subject of human interest.

But, the concept of DNA fingerprinting is totally a new approach in the field of molecular biology. Sir Alec Jeffreys (1985-86) invented the DNA fingerprinting technique at Leicester University, United Kingdom.

Meaning:

DNA of an individual carries some specific sequence of bases, which do not carry any information for protein synthesis. Such nucleotide base sequences are repeated many times and are found in many places throughout the length of DNA. The number of repeats is very specific in each individual. The tandem repeats of short sequences are called ‘mini satellites’ or ‘variable number tandem repeats’ (VNTRs). Such repeats are used as genetic markers in personal identity.

Technique:

1. The first step is to obtain DNA sample of the individual in question.
2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim’s cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.
3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.
4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.
5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.
6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes. These probes contain repeated sequences of bases complimentary to those on mini satellites.
7. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.
8. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to

the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.
9. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.
10. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy

Significance:

1. The technique is extensively used as confirmatory test in crime detection in cases of rape and murder.
2. Disputed parentage can be solved by the technique.
3. This method can confirm species of more closeness or far apart from evolutionary point of view so that taxonomical problems can be solved.
4. The technique also can be used to study the breeding pattern of endangered animals.
5. Clinically this method can be used in restoring the health of blood cancer patients.

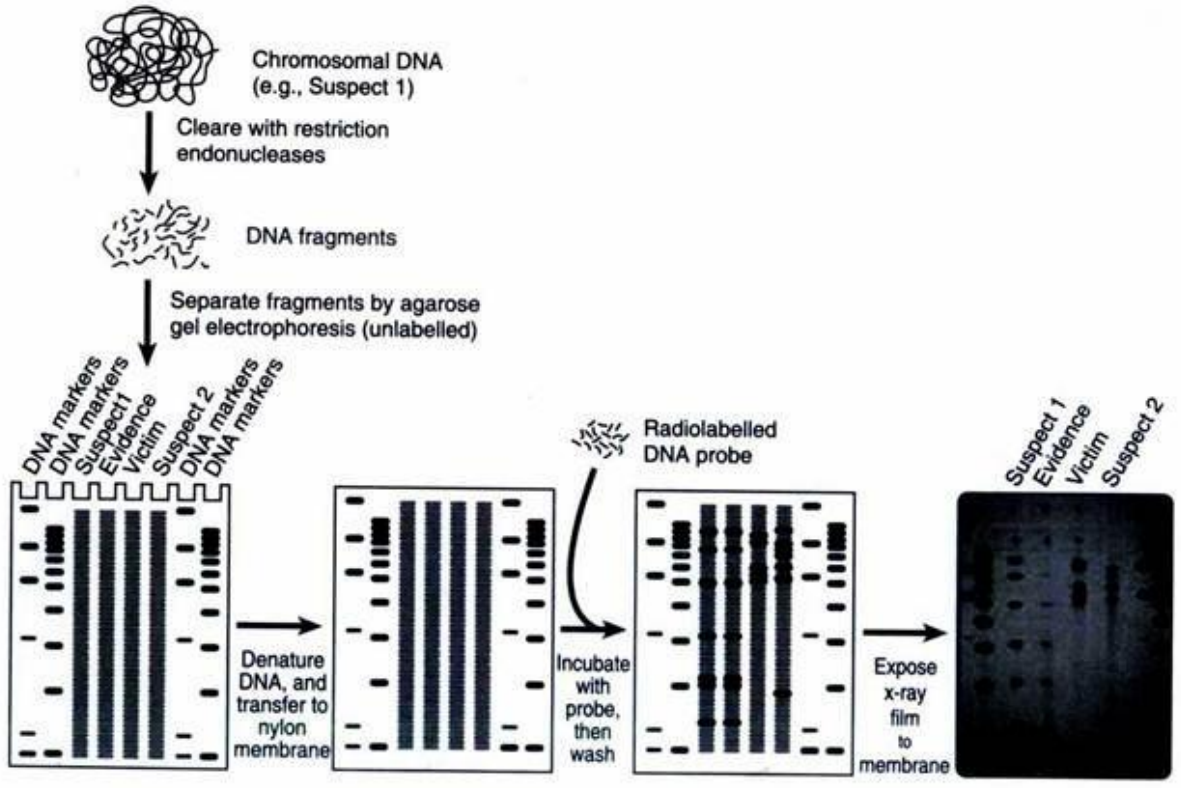


Fig. 4.21: DNA fingerprinting procedure

E. SOUTHERN BLOTTING TECHNIQUE

History

Edwin Southern, the inventor of Southern blotting started a trend to his invention after him. It was introduced as a technique to detect particular sequence of DNA in DNA samples. He first published the article in 1975.

Southern integrated three innovations to create the Southern blot – restriction endonucleases, gel electrophoresis and blotting through methods.DNA fragments were differentiated using electrophoresis based on size, then transferred to a membrane and hybridized with a radio labeled DNA probe.

Definition

An example of RFLP(restriction fragment length polymorphism), southern blotting can be defined as an analytical technique for identifying specific sequences of DNA by separating fragments on a gel and transferring them to a second medium (carrier membrane) on which hybridization testing may be carried out. During southern blotting, the DNA fragments are immobilized as a result, the membrane carries a semi-permanent reproduction of the banding pattern of the gel. The DNA are then exposed to hybridization analysis allowing bands with sequence resemblance to a labeled probe to be identified.

There are different types of membrane, transfer buffer and transfer methods to set up a southern blot. The most common and popular membranes are made of nitrocellulose, uncharged nylon positively charged nylon but they are interchangeable depending on the applications.

Principle

It is based on the principle of transfer of separated DNA fragments to a carrier membrane (usually nitrocellulose) using gel electrophoresis and subsequent identification of specific DNA fragmentsby labelled probe hybridization. Hybridization is a technique in which a double stranded DNA molecule is formed in between a single stranded DNA probe and a target single stranded DNA. The probes are labeled with a marker and complementary to the target DNA as a result we can detect one molecule of target in a mixture of millions after hybridization as the reactions are specific.

Procedure

1. Extract and purify DNA from cells
   - We separate the DNA to be tested from the rest of the cellular material in the nucleus.

- We then incubate the specimen with detergent to promote cell lysis (frees cellular proteins and DNA).
- Proteins are removed through organic and non-organic extraction.
- We then use alcohol precipitation to purify the DNA from the solution.
- Visible DNA fibers are removed and suspended in buffer.

2. DNA fragmentation

- We use restriction endonuclease enzyme to break long nucleotide sequence into smaller fragments for purification or identification process.
- Each restriction enzymes are validated with universal buffers (L, M, H, K, or T (+BSA)) and supplied with recommended buffer.
- Before appropriate DNA concentration and establishing a restriction digestion with preferable enzymes, we keep reagent necessary for digestion process on ice.
- The components are then added into a PCR tube and mixedby absorbing the contents with the help of pipette slowly avoiding formation of any bubbles.
- PCR amplifies the number of fragments of DNA obtained from the restriction digest which are easily separated using gel electrophoresis.

3. Gel electrophoresis

- Sorts the complex mixture of DNA fragments according to size.
- The percentage and size of the gel to be used must be determined.
- Gels consist of microscopic pores and are solid (usually agarose or polyacrimide).
  Generally, 0.7 – 2% gel is considered to be adequate for most of the applications.
- Nucleic acids have negative charge and move from left to right. The large molecules are held up while smaller ones move faster causing separation by size.
- Gels are stained with ethidium bromide to permit photography under UV light.
- A single band form is given to intact high quality DNA (small amount of degradation is tolerable).

4. Denature DNA

- DNA obtained are double stranded in nature.
- Alkalis are used to denature the restriction fragments in the gel that makes double stranded DNA to become single stranded.

- To avoid re-hybridization, we use NaCl so that DNA is neutralized.

5. Blotting

- Transfer DNA from the gel to solid support (carrier membrane).
- We dry the blot (around 80°C) or use UV radiation to make it permanent.

6. Hybridization

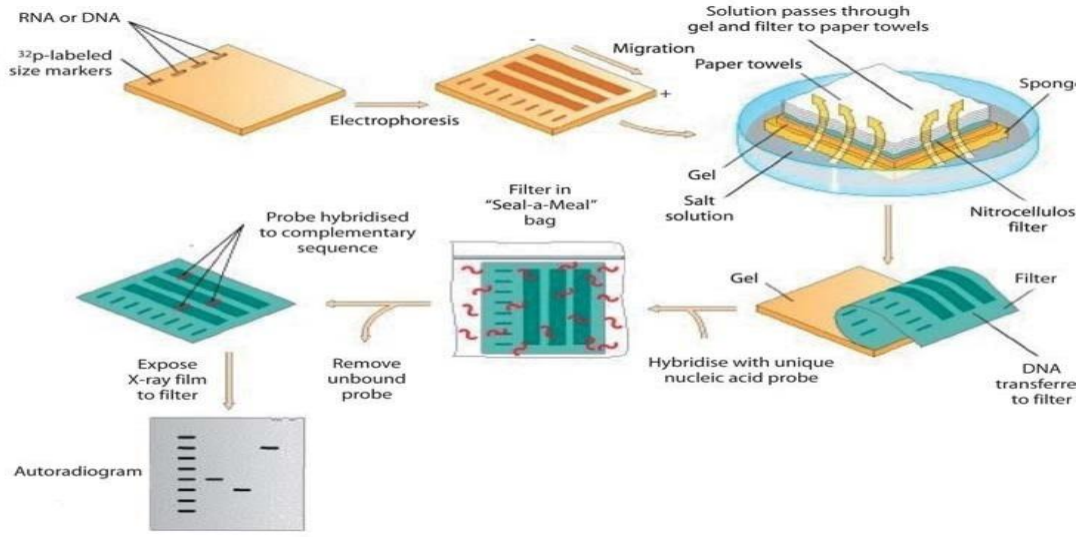
- The membrane bounded with DNA are incubated after adding the labelled probe.
- Usually requires 1-16 hours depending on the complexity of the probe and concentration.
- The probe then binds with complementary DNA on the membrane with the help of BSA or casein (blocks all other non-specific binding).

7. Washing

- Despite using blocking agents, some excess probe binds to the membrane.
- Wash buffers containing NaCl and detergent washes away the excess probe.

8. Autoradiography

- The particles are exposed to X-ray film when we use radioactive probe or fluorescent probe.
- If a chromogenic detection method is used, we can see development of color on the membrane.



Southern Blotting

Applications

- Identify specific DNA from a DNA sample.
- Identification of viral and bacterial infections.
- Important in the study of gene mutation, deletion and rearrangements.
- DNA fingerprinting (maternity and paternity analysis, forensic studies and personal identification).
- Diagnosis of neonatal and genetic diseases including cancer.
- Discovery of RFLP (restriction fragment length polymorphism) to map crucial genomes.

Advantage and Disadvantages

Advantages

- Less degraded compared with protein and mRNA as DNA are very stable.
- Effective way to detect specific DNA sequence from large complex samples.
- Increased sensitivity of fragments detection because of probe label used for amplifying signals.
- Only way to diagnose FSHD (Facioscapulohumerals Muscular Dystrophy).

Disadvantages

- Not applicable in routine diagnostic setting.
- Time consuming and requires large amount of DNA.
- Does not allow morphologic preservation of tissue so historic evaluation features are not available.

F.WESTERN BLOTTING

Definition

Western blotting is a widely used technique in molecular biology and immuno-genetics for the detection and analyses of proteins.

This method is also called “immune blotting” because of its nature to use an antibody for specifically identifying its antigen and also protein blotting.

A qualitative and semi quantitative data can be produced using western blotting for the desired protein of interest.

Principle

- Western blotting technique principle relies on the specificity of binding between a molecule of interest and a probe to allow detection of the molecule of interest in the mixture of many other similar molecules.
- Here, molecule of interest is protein whereas the probe is typically an antibody raised against that particular protein.
- SDS PAGE is a prerequisite for western blotting .
- Proteins get spilt up by their size by a process called SDS-polyacrylamide gel electrophoresis.
- A primary antibody (an antibody precisely for the target protein) is then used to probe and wash the membrane with transferred protein.
- This primary antibody treated membranes are then reacted with a secondary antibody, usually an antibody enzyme conjugate (e.g. horseradish peroxidase).
- The target protein is visualized as band on blotting paper, X-ray film or imaging system.

Procedure

1. **Sample Preparation**

- Use different samples to extract protein(e.g. tissues or cells)
- Use homogenizer or sonication to breakdown samples.
- Prevent sample digestion at cold temperature through protease and phosphates.
- Finally, observe concentration of proteins and use spectrophotometer for protein concentration.

2. Gel Electrophoresis

- Proteins are separated on the basis of their size, shape and charge.

- In SDS gel electrophoresis, protein samples are separated according to their molecular weights.
- Load protein samples to polyacrylamide gel (*Higher percentage of gels are used for low molecular weight problems and vice versa*).

3. Protein Transfer

- A solid support membrane is placed where we transfer the separated protein for antibody detection.
- The method is called electro blotting where an electric field is directed perpendicular to the surface of the gel maintain their relative position.
- A transfer sandwich is created made up of: a fiber pad (sponge), filter papers, blotting membrane, gel, filter papers and finally a fiber pad.

4. Protein staining

- The gels must be stained as proteins are not directly visible in the gel.
- Dyes like coomassie blue, silver stain or deep purples are used.
- The gel is imaged with suitable instrument and a permanent record can be made after staining.

5. Blocking

- Prevents non-specific binding of antibodies by blocking unoccupied sites of membrane with inert protein or non-ionic detergent.
- BSA and non-fat dry milk are commonly used typical blockers.
- Blocking agents should possess greater connection towards membrane than the antibodies.

6. Antibody probing

- Incubate the blot with one or more antibodies
- Primary antibodies are specific depending on the antigens to be detected.
- The secondary antibody (monoclonal or polyclonal) is linked to an enzyme that is used to indicate the location of the protein.

7. Washing

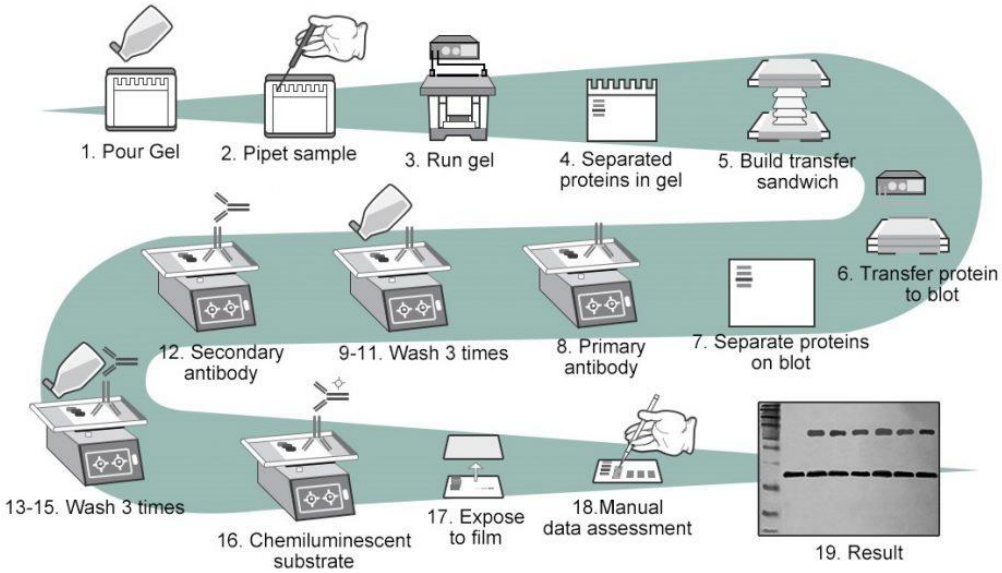
- Removes unbounded antibodies from the membrane.
- Commonly used buffer : a dilute solution of tween-20 in TBS or PBS buffer .

8. Protein Detection

- Alkaline phosphates (AP) and horse radish peroxidase (HRP) are widely used.
- Four types: Chromogenic detection, Chemiluminescence detection, Fluorescent detection and radioactive detection.

9. **Analysis and Imaging**

- Detection of signals using X-ray film, scanners or CCD.
- Benchmarking with marker protein to estimate the molecular weight of the protein.
- Verification can be done through qualitative and quantitative analysis to show the presence and absence of specific proteins of interests.



Western Blotting

Applications

- Detection of particular protein from a mixture of proteins.
- Size and amount estimate of proteins in the mixture.
- Verification following a high sensitivity ELISA test for diagnosis of Lyme, HIV infection, BSE, HBV and so on.
- Detect condensed isoforms of proteins as well as tagged proteins.

Advantage and Disadvantages

Advantages

- Effective early diagnostic tool.
- Detect minimal immunogenic response form virus or bacteria.
- Requires fewer antibodies for testing.

- Detect specific protein from a large mixture of different proteins. (Even more than 300,000)

Disadvantages

- Requires specific primary antibodies to perform test on desired protein of interest.
- Challenging and hence requires well trained staffs.
- Poorer results as antibodies may revel off-target bindings.
- Detecting and imaging the results can be expensive as equipment cost is high.

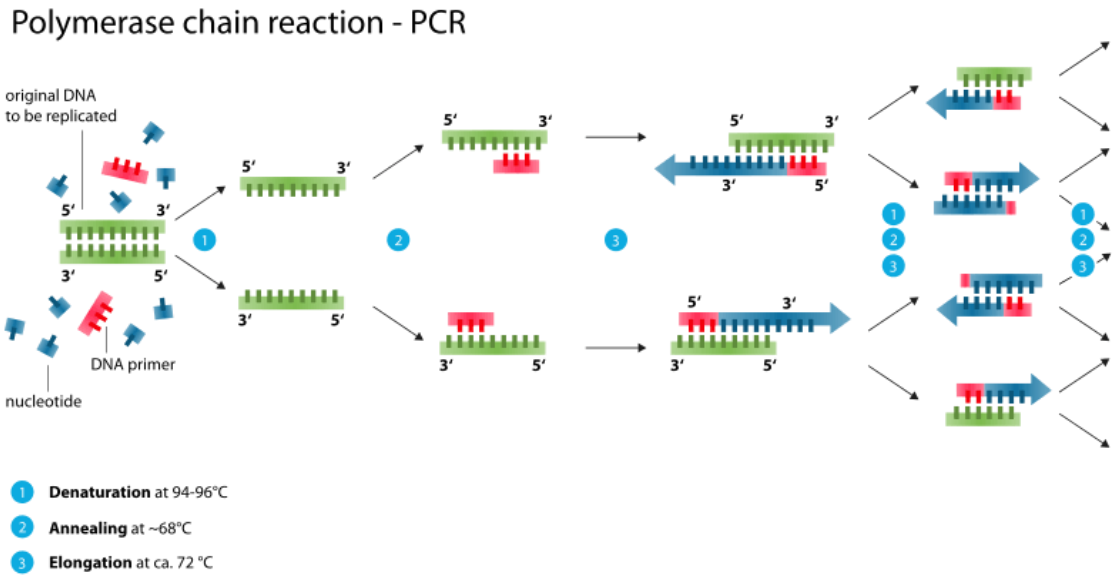
2. POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. This procedure is carried out entirely biochemically, that is, in vitro. PCR was invented by Kary Mullis in 1983. He shared the Nobel Prize in chemistry with Michael Smith in 1993.

Principle of PCR

PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Procedure/Steps of PCR



Polymerase

1. Denaturation

The DNA template is heated to 94° C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing

The mixture is cooled to anywhere from 50-70° C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

With one cycle, a single segment of double-stranded DNA template is amplified into two separate pieces of double-stranded DNA. These two pieces are then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template is increased exponentially.

Types of PCR

1. Real-time PCR
2. Quantitative real time PCR (Q-RT PCR)
3. Reverse Transcriptase PCR (RT-PCR)
4. Multiplex PCR
5. Nested PCR
6. Long-range PCR
7. Single-cell PCR
8. Fast-cycling PCR
9. Methylation-specific PCR (MSP)
10. Hot start PCR
11. High-fidelity PCR
12. In situ PCR
13. Variable Number of Tandem Repeats (VNTR) PCR
14. Asymmetric PCR
15. Repetitive sequence-based PCR
16. Overlap extension PCR
17. Assemble PCR
18. Inter sequence-specific PCR(ISSR)
19. Ligation-mediated PCR
20. Methylation –specifin PCR
21. Mini primer PCR
22. Solid phase PCR
23. Touchdown PCR, etc

Applications of PCR

1. PCR is used in analyzing clinical specimens for the presence of infectious agents, including HIV, hepatitis, malaria, anthrax, etc.
2. PCR can provide information on a patient's prognosis, and predict response or resistance to therapy. Many cancers are characterized by small mutations in certain genes, and this is what PCR is employed to identify.
3. PCR is used in the analysis of mutations that occur in many genetic diseases (e.g. cystic fibrosis, sickle cell anaemia, phenylketonuria, muscular dystrophy).
4. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required, for example, sufficient DNA can be obtained from a droplet of blood or a single hair.
5. PCR is an essential technique in cloning procedure which allows generation of large amounts of pure DNA from tiny amount of template strand and further study of a particular gene.
6. The Human Genome Project (HGP) for determining the sequence of the 3 billion base pairs in the human genome, relied heavily on PCR.
7. PCR has been used to identify and to explore relationships among species in the field of evolutionary biology. In anthropology, it is also used to understand the ancient human migration patterns. In archaeology, it has been used to spot the ancient human race. PCR commonly used by Paleontologists to amplify DNA from extinct species or cryopreserved fossils of millions years and thus can be further studied to elucidate on.

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PROPOSED QUESTION PAPER PATTERN

Q.NO:1: IMMUNOLOGY EXPERIMENT-------7 MARKS

(I-1,2,3)

1. **AGGLUTINATION TEST**
2. **VDRL TEST**
3. **RADIAL IMMUNODIFFUSION TEST**

Q.NO:2: BIOTECHNOLOGY EXPERIMENT---7 MARKS

(II-1-c,d,e,f & 2)

II-1.C: DNA SEQUENCING

D: DNA FINGER PRINTING

E: SOUTERN BLOTTING

F: WESTERN BLOTTING

II-2: POLYMERASE CHAIN REACTION

Q.NO:3: 3.SPOTTERS 6 MARKS

(I-4, II-a,b)

I-4: a. SPLEEN b. THYMUS c. LYMPHNODE d. BONE MARROW

II-a. i. PLASMID ii. BACTERIOPHASE iii. COSMID

II.b. i. TRANSGENIC MICE ii. TRANSGENIC FISH iii. T.COW

Q.NO:4: RECORD 5 MARKS