Shree H. N. Shukla Institute of Pharmaceutical Education and Research, Rajkot

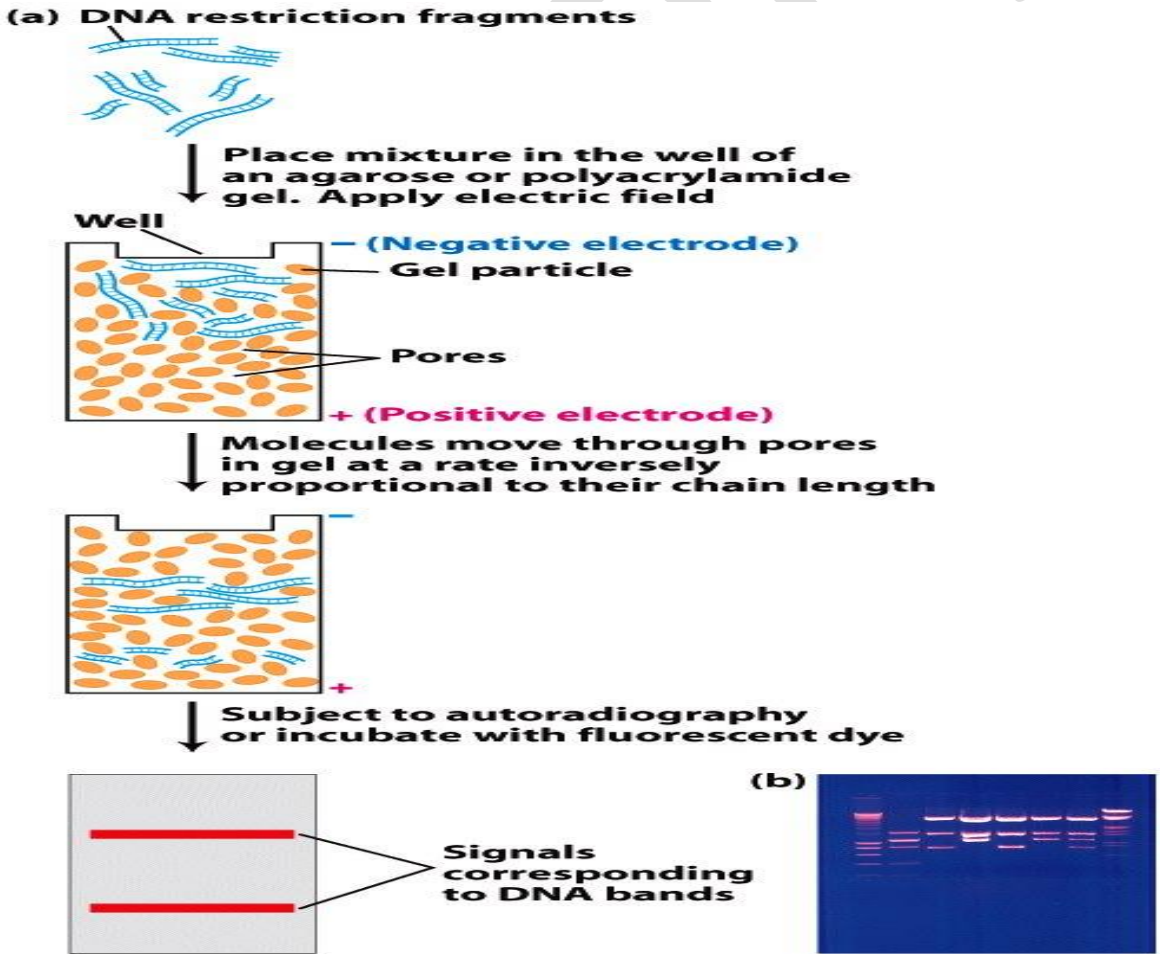
B. Pharm
Semester-V

Subject Name: Pharmaceutical Biotechnology
Subject Code: BP505TT

Chapter-4
IMMUNO BLOTTING TECHNIQUES

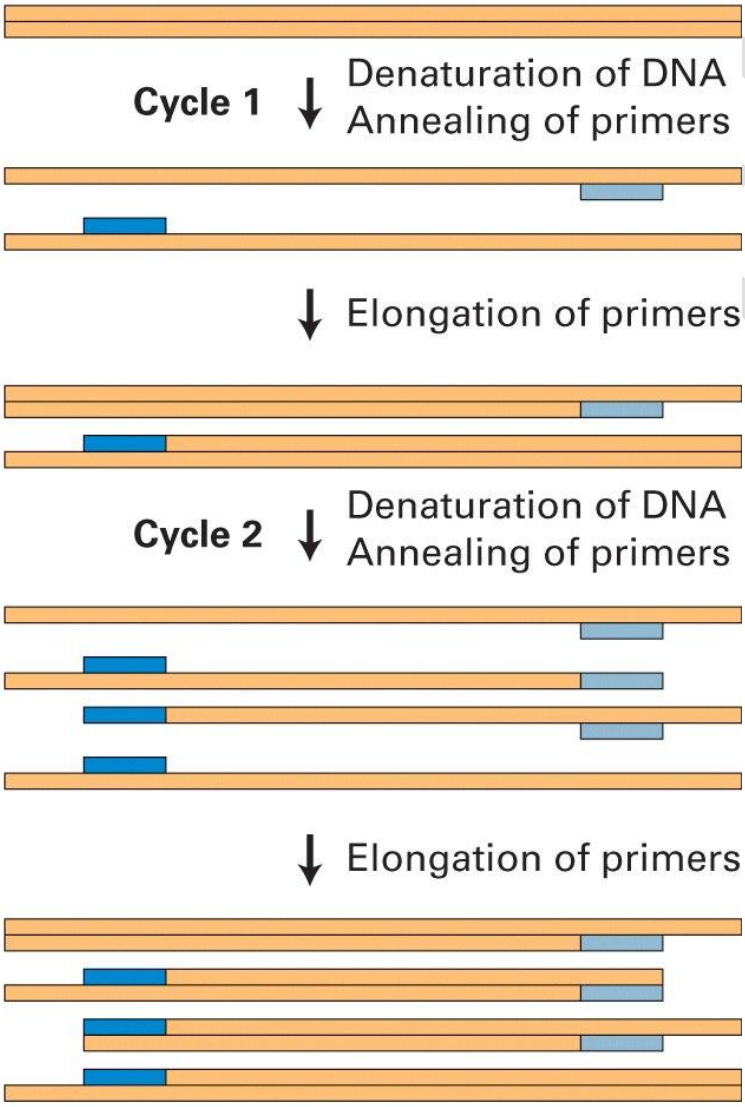
Separation of DNA Fragments by Gel Electrophoresis

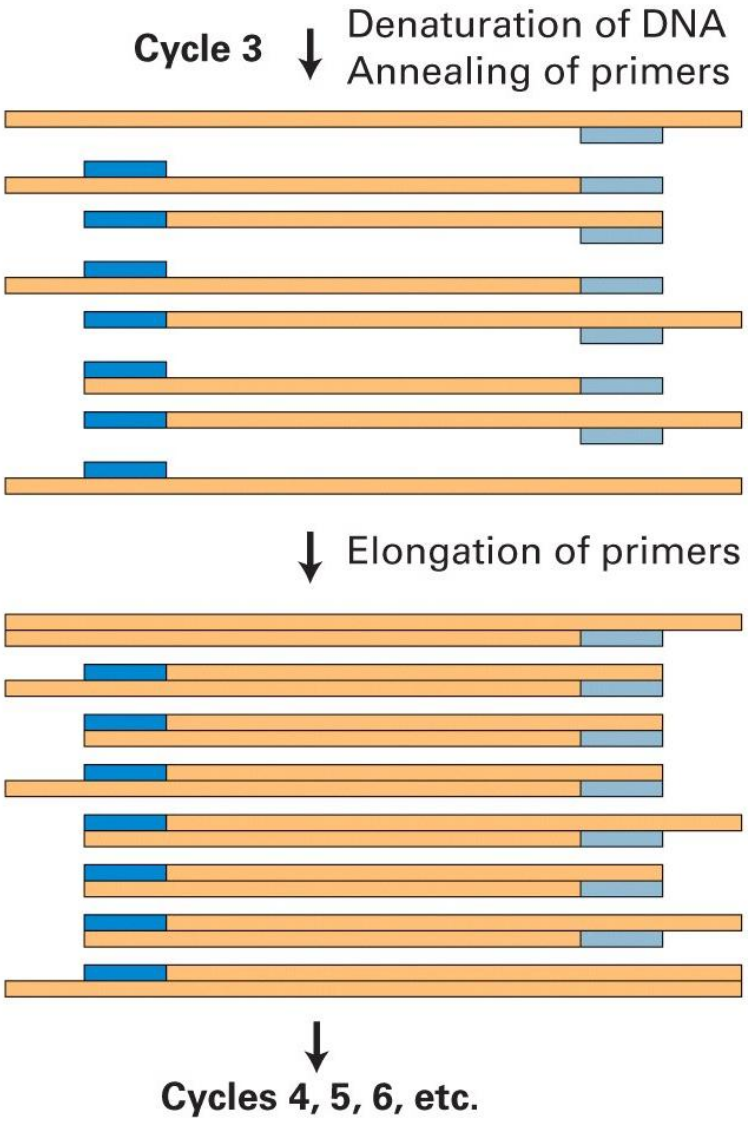
- DNA fragments must be separated and purified prior to many rDNA procedures. A convenient method for both separation and purification is gel electrophoresis.
- DNA molecules have a uniform charge to mass ratio. In an electric field they run towards the anode and are separated based on size (length) when electrophoresed through a gel sieving network made of polyacrylamide or agarose .
- DNA bands can be visualized by radiolabeling the DNA or by noncovalent binding of the fluorescent dye known as ethidium bromide.
- The region of the gel containing the band can be excised and the DNA fragment obtained by extraction with a buffer.



Polymerase Chain Reaction

- The PCR is a method for amplifying a DNA sequence region located between two primers (Fig).
- Amplification is specific and highly sensitive, allowing a target sequence to be specifically amplified starting from a complex mixture of DNA.
- In Cycle 1, double-stranded DNA containing the target sequence is first denatured by heating to >90˚C, PCR primers are annealed by reducing the temperature to ~ 50-60˚C, and then the primers are elongated by a DNA polymerase.
- The process is repeated over many cycles.

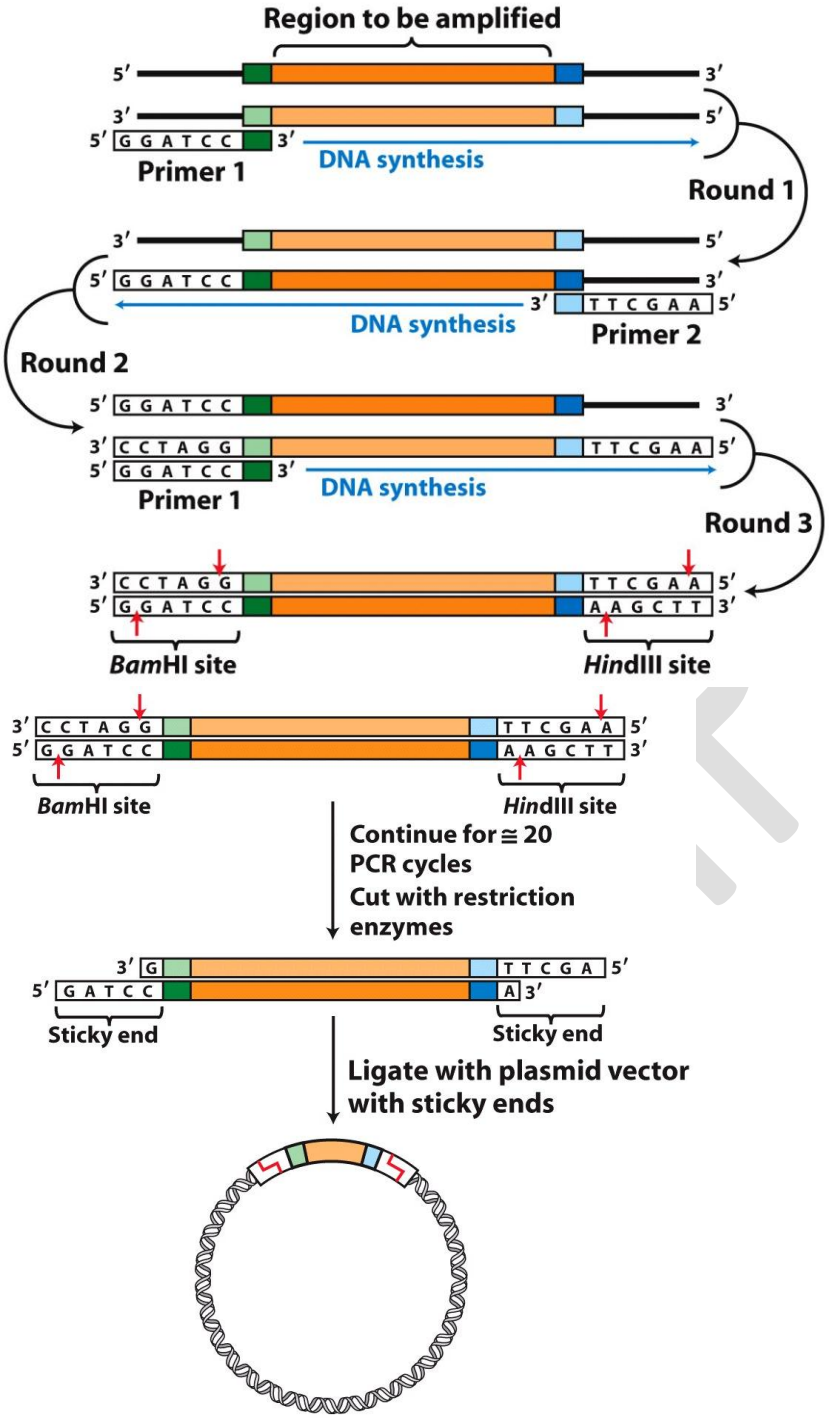




- In later cycles, most of the DNA synthesized corresponds exclusively to the sequence region between the primers.
- The yield of amplified DNA increases exponentially based on the cycle number, n (yield is proportional to 2^n). A heat-resistant DNA polymerase, typically *Taq* polymerase from the Yellowstone Archaen organism, *Thermus aquaticus*, is used as the DNA polymerase to prevent its denaturation due to heating.

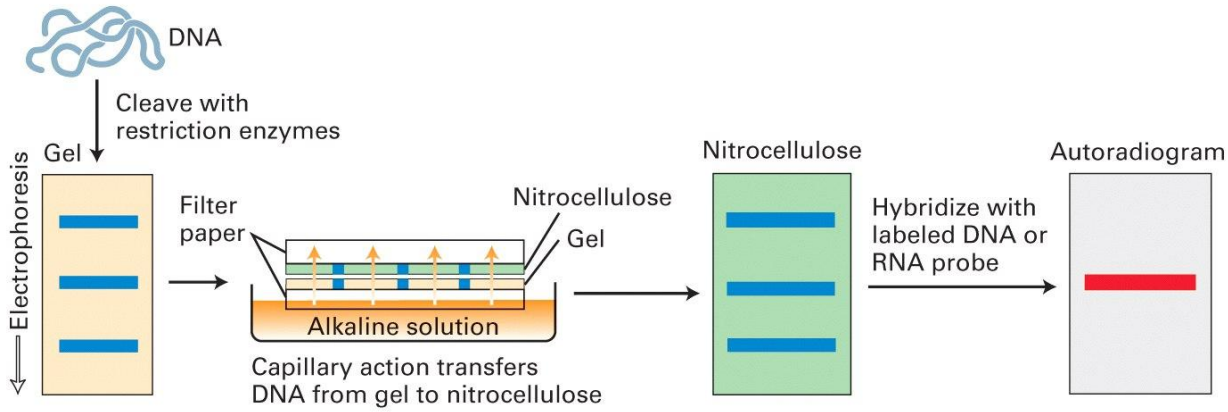
Cloning of PCR-amplified DNA

- PCR fragments can be readily cloning into a vector by incorporating restriction enzyme sites into the ends of the primers used in amplification
- The added sequences do not interfere with polymerization reactions.


Region to be amplified
5′
3′
3′
5′
G G A T C C
3′
Primer 1
DNA synthesis
Round 1
3′
5′
5′
G G A T C C
3′
T T C G A A
5′
3′
DNA synthesis
Primer 2
Round 2
5′
G G A T C C
3′
3′
C C T A G G
T T C G A A
5′
5′
G G A T C C
3′
Primer 1
DNA synthesis
Round 3
3′
C C T A G G
T T C G A A
5′
5′
G G A T C C
A A G C T T
3′
BamHI site
HindIII site
3′
C C T A G G
T T C G A A
5′
5′
G G A T C C
A A G C T T
3′
BamHI site
HindIII site
Continue for ≅ 20
PCR cycles
Cut with restriction
enzymes
3′
G
T T C G A
5′
5′
G A T C C
A
3′
Sticky end
Sticky end
Ligate with plasmid vector
with sticky ends

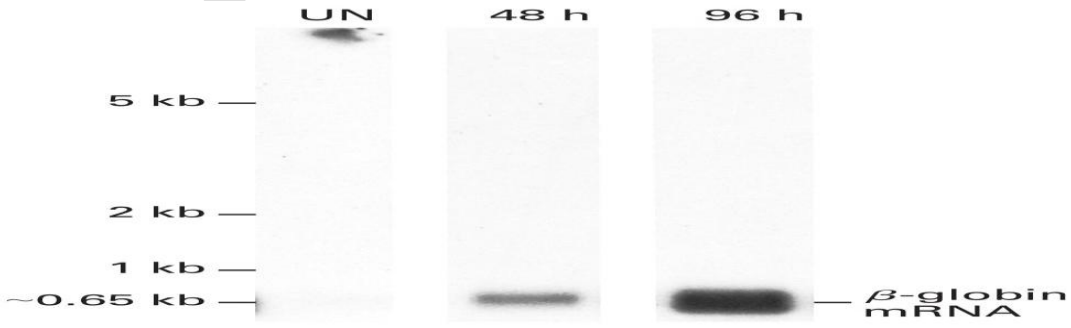
Southern Blotting

- Southern blotting is a sensitive method for the detection of a DNA sequence within a mixture (Fig.)
- The DNA first is cleaved with a restriction enzyme to produce fragments that can be separated by electrophoresis.
- After electrophoresis, DNA fragments are denatured with alkali and transferred to a nitrocellulose membrane by capillary action, creating a replica of the original gel.
- The membrane is incubated with a labeled probe which binds to and detects the fragment of interest.



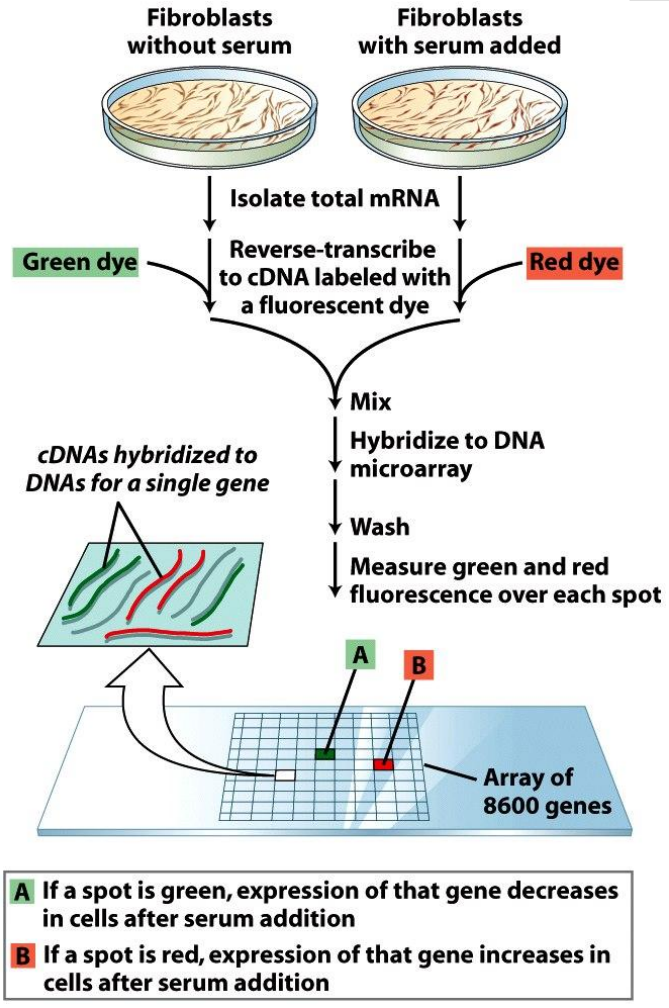
Northern Blotting

- Northern blotting is a method for the detection of a specific mRNA in a mixture of RNAs. Like Southern blotting the method is highly specific and sensitive.
- RNA from a cell/tissue is extracted and separated by electrophoresis.
- As in Southern blotting the RNA is transferred to a nitrocellulose membrane and incubated with a labeled probe that is complementary to the RNA.
- As shown in (Fig) the method can be use to quantitate transcription of a mRNA under different cellular conditions.



DNA Microarrays and Transcriptome Analysis

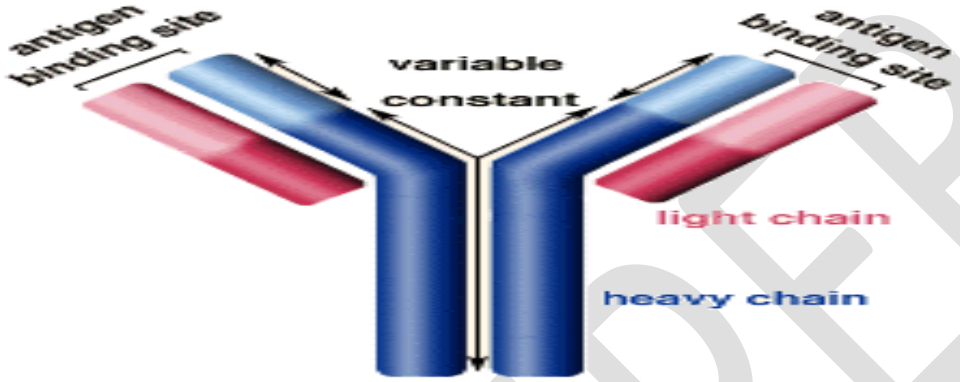
- A complete analysis of all the mRNAs transcribed in an organism (transcriptome) can be performed by DNA microarray analysis (Fig.).
- In this method, mRNA is isolated and converted to cDNA, and then labeled with a fluorescent dye.
- The cDNA is hybridized to a gene chip containing oligonucleotide sequences representing all or a subset of genes in the organism.
- The amount of mRNA expressed from each gene is determined by quantitation of fluorescence intensity of the cDNA bound to each probe.
- The method can be adapted to compare gene expression levels in cells under different growth conditions, etc



ELISA

Enzyme Linked Immunosorbent Assay

- Antibodies (also known as immunoglobulins abbreviated Ig) are gamma globulin proteins that are found in blood and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses.

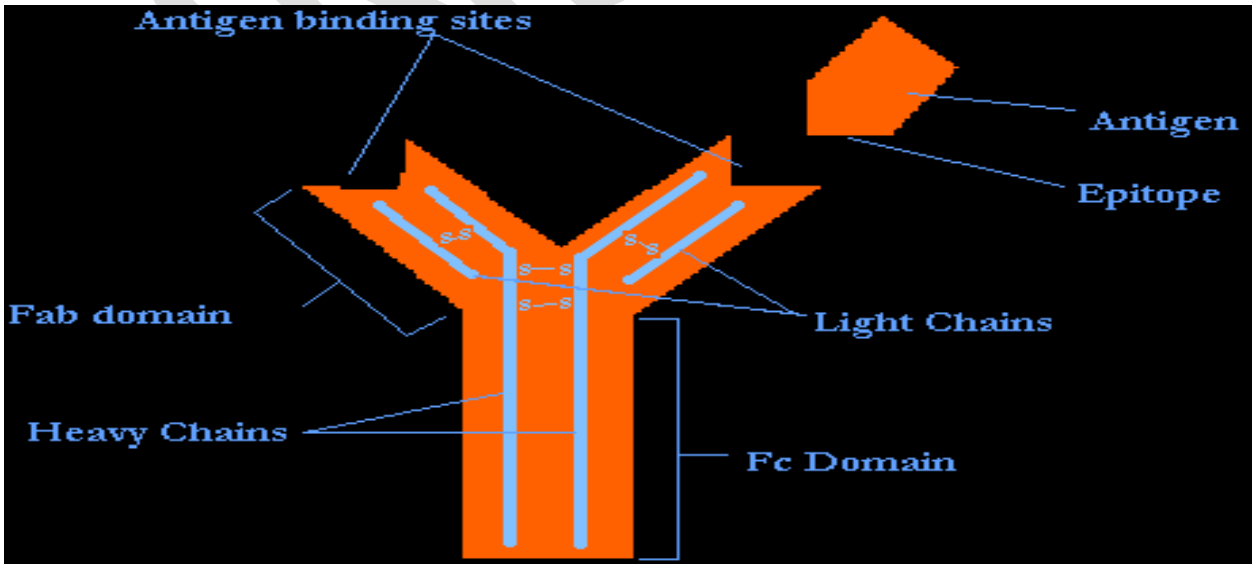


- **Antigens**

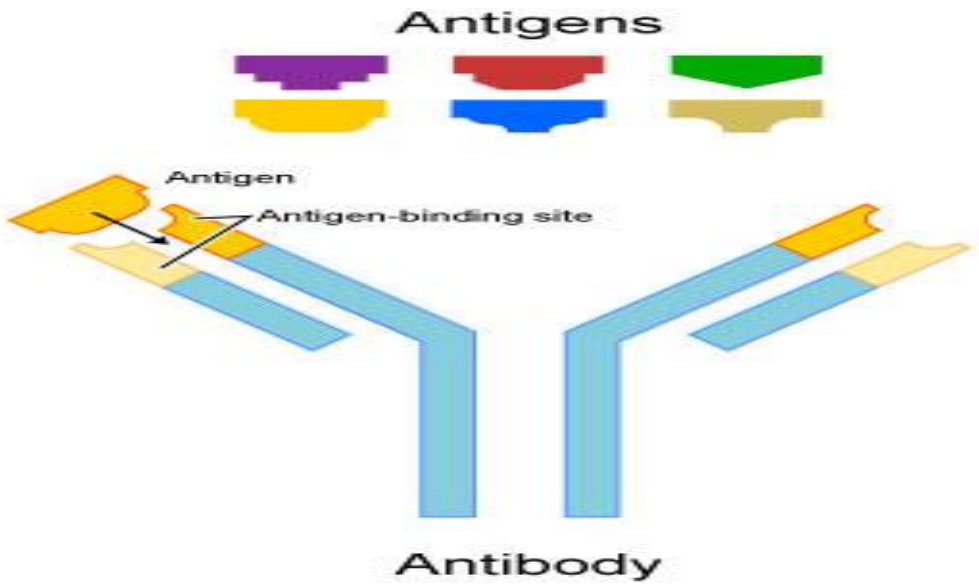
A substance that when introduced into the body stimulates the production of an antibody

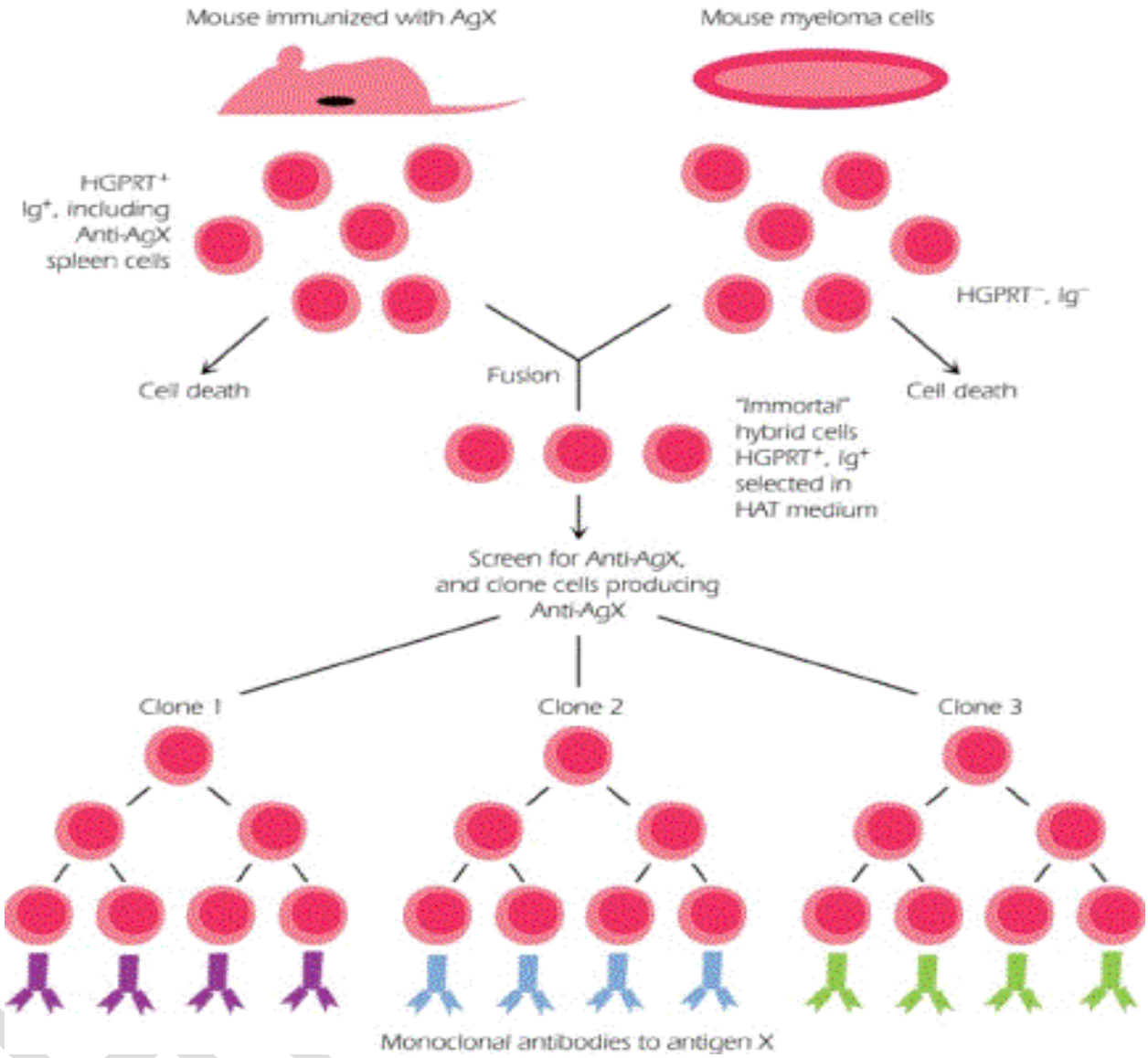
- **Immunoassay**

A laboratory technique that makes use of the binding between an antigen and its homologous antibody in order to identify and quantify the specific antigen or antibody in a sample.



- Is present naturally in the body like hormones
- Is manufactured in special disease status for example human chorionic gonadotrophin hormone (HCG) which is normally produced by cells of the placenta in pregnancy is found in the body in some types of cancer
- Is not present in the body in normal condition like drugs.
- **The Antibody:** An immunoglobulin, a specialized immune protein, produced because of the introduction of an antigen into the body, and which possesses the remarkable ability to combine with the very antigen that triggered its production (specific affinity)
- The antibody recognises and bind to the antigenic determinant region of the antigen.
- Specific antibodies are produced by injecting an antigen into a mammal, such as a mouse, rat or rabbit for small quantities of antibody, or goat, sheep, or horse for large quantities of antibody.
- Blood isolated from these animals contains *polyclonal antibodies*—multiple antibodies that bind to the same antigen—in the serum, which can now be called antiserum.
- To obtain antibody that is specific for a single antigen, antibody-secreting lymphocytes are isolated from the animal and immortalized by fusing them with a cancer cell line.
- The fused cells are called hybridomas, and will continually grow and secrete antibody in culture.
- Single hybridoma cells are isolated by dilution cloning to generate cell clones that all produce the same antibody; these antibodies are called *monoclonal antibodies*





Is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.

- The technique is divided into

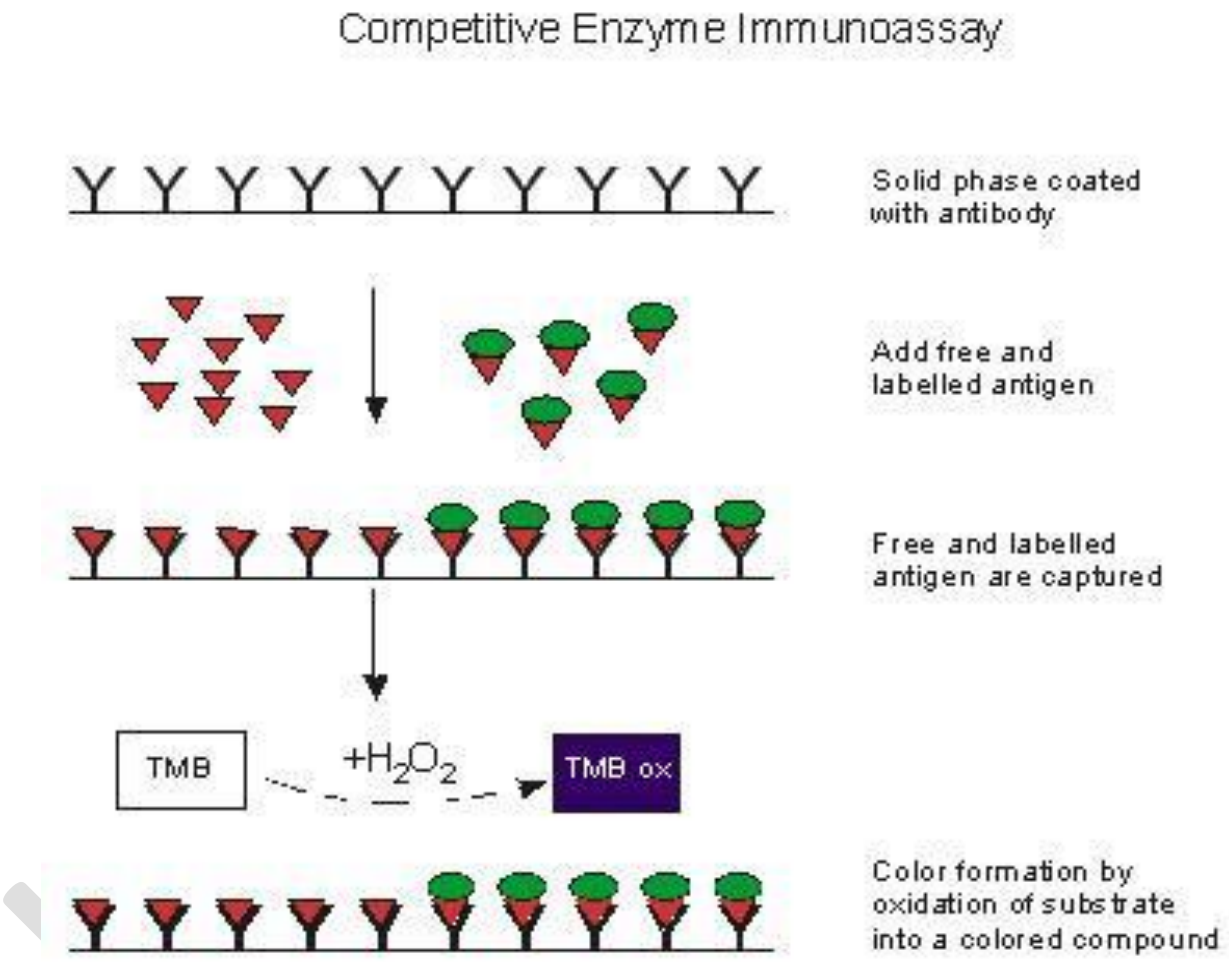
  1- Competitive ELISA

  2- Sandwich ELISA (also called direct ELISA)

  3- Indirect ELISA

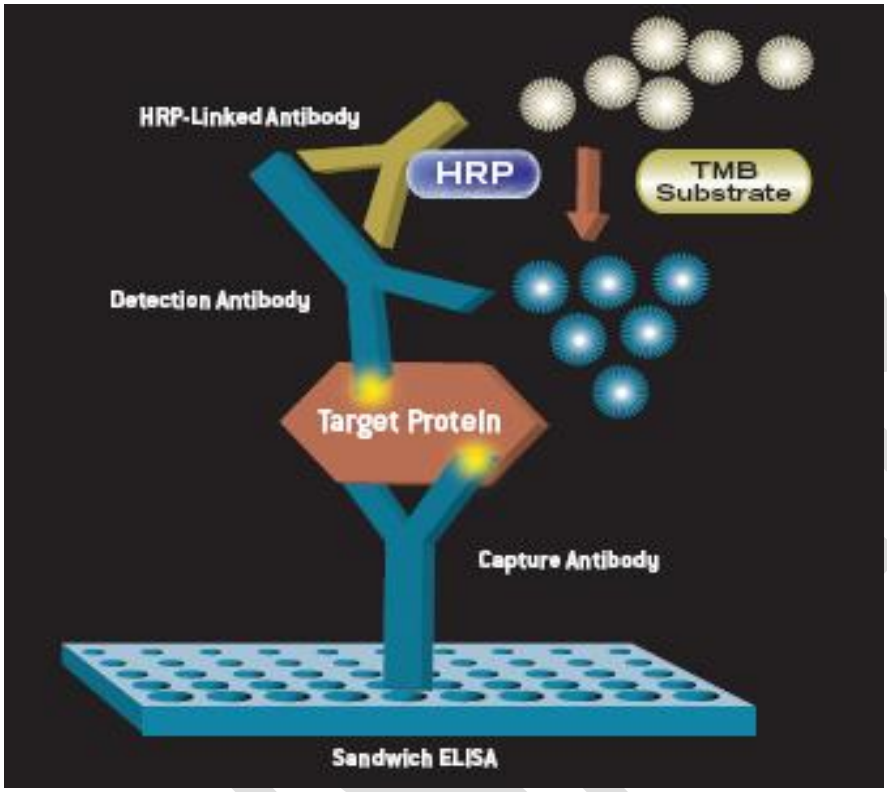
Competitive ELISA

- The labelled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The more antigen in the sample, the less labelled antigen is retained in the well and the weaker the signal).



Sandwich ELISA

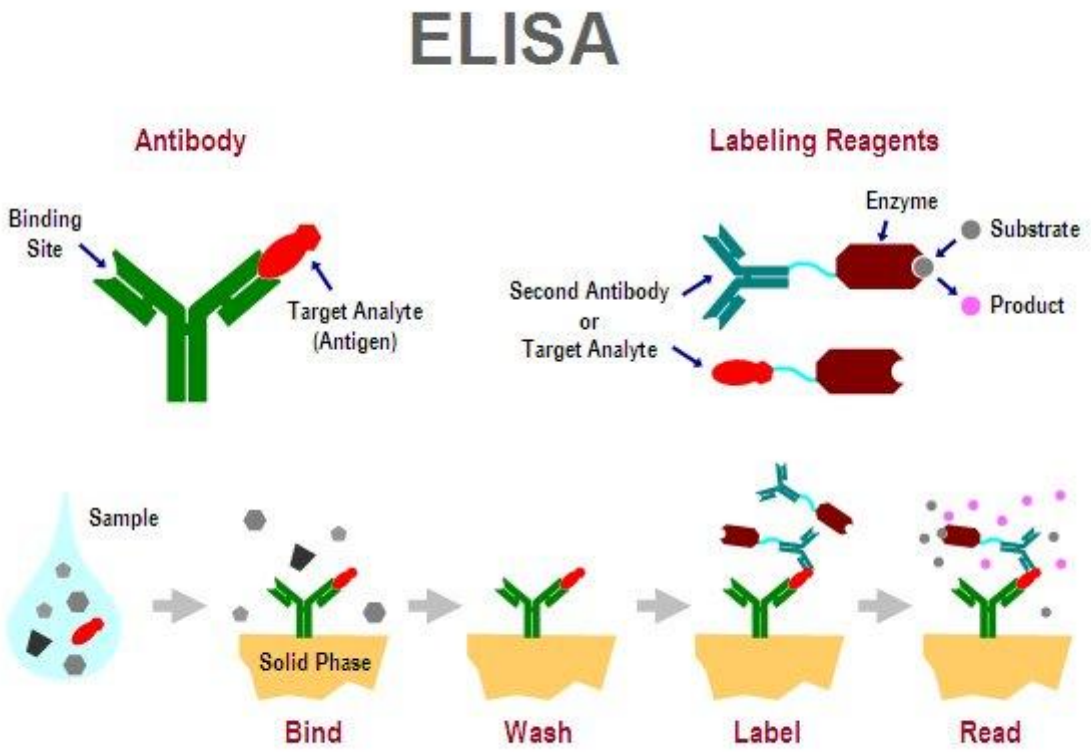
The ELISA plate is coated with Antibody to detect specific antigen.



- Prepare a surface to which a known quantity of capture antibody is bound.
- Block any non specific binding sites on the surface
- Apply the antigen-containing sample to the plate.
- Wash the plate, so that unbound antigen is removed.
- Apply enzyme linked primary antibodies as detection antibodies which also bind specifically to the antigen.
- Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
- Apply a chemical which is converted by the enzyme into a coloured product.

- Measure the absorbency of the plate wells to determine the presence and quantity of antigen

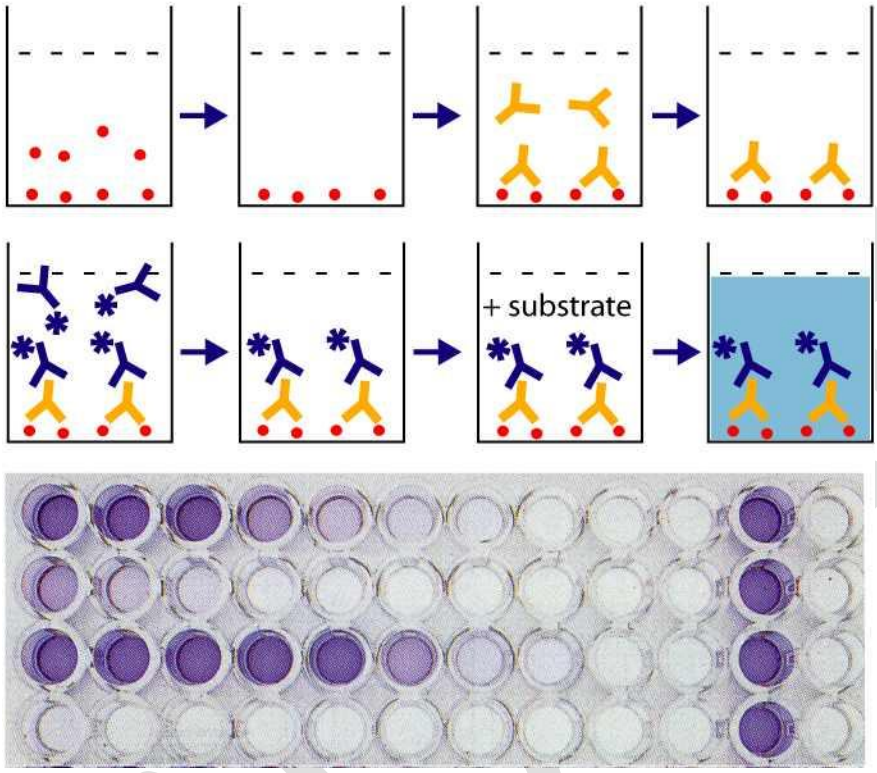
Indirect ELISAIndirect ELISAIndirect ELISA



Indirect ELISA

- The protein antigen to be tested for is added to each well of ELISA plate, where it is given time to adhere to the plastic through charge interactions.
- A solution of non-reacting protein is added to block any plastic surface in the well that remains uncoated by the protein antigen.
- Then the serum is added, which contains a mixture of the serum antibodies, of unknown concentration, some of which may bind specifically to the test antigen that is coating the well.
- Afterwards, a secondary antibody is added, which will bind to the antibody bound to the test antigen in the well. This secondary antibody often has an enzyme attached to it.
- A substrate for this enzyme is then added. Often, this substrate changes colour upon reaction with the enzyme. The colour change shows that secondary antibody has bound to primary antibody, which strongly implies that the donor has had an immune reaction to the test antigen.
- The higher the concentration of the primary antibody that was present in the serum, the stronger the colour change. Often a spectrometer is used to give quantitative values for colour strength.

Indirect ELISA



- Before starting the work read kit instruction carefully
- 1- The 96 well plate is labeled carefully and the first wells are used to draw the standard curve.
- The sample is added to plate in duplicate or triplicate and then the mean result is calculated.
- The quality control sample which is provided with the kit is treated as the test samples.