RAMA UNIVERSITY

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FACULTY OF AGRICULTURE SCIENCES AND ALLIED INDUSTRIES

(Principles of Biotechnology)

For

M.Sc. Ag (GPB)

Course Instructor

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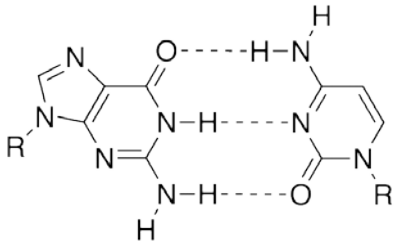
METHODS OF NUCLEIC ACID HYBRIDIZATION

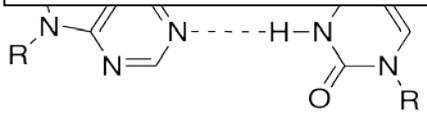
3-4.1 Introduction:

Nucleic acid hybridization is a basic technique in molecular biology which takes advantage of the ability of individual single-stranded nucleic acid molecules to form double-stranded molecules. According to Watson-Crick base pairing, adenine binds with thymine and guanine binds with cytosine by hydrogen bonding.



Guanine Cytosine

A GC base pair demonstrating three intermolecular hydrogen



Adenine Thymine

An AT base pair demonstrating two intermolecular hydrogen bonds

3-4.2 Southern hybridization:

The basic principle behind the southern hybridization is the nucleic acid hybridization. Southern hybridization commonly known as southern blot is a technique employed for detection of a specific DNA sequence in DNA samples that are complementary to a given RNA or DNA sequence. It was the first blotting technique to be devised, named after its pioneer E.M Southern, a British biologist. Southern blotting involves separation of restricted DNA fragments by electrophoresis and then transferred to a nitrocellulose or a nylon membrane, followed by detection of the fragment using probe hybridization.

Separated by electrophoresis is transferred from gel to a membrane which in turn is used as a substrate for hybridization analysis employing labeled DNA or RNA probes specific to target fragments in the blotted DNA. Southern hybridization helps to detect specific fragment against a background of many other restriction fragments. Southern blotting is a technique which is used to confirm the identity of a cloned fragment or for recognition of a sub-fragment of interest from within the cloned DNA, or a genomic DNA. Southern blotting is a prerequisite to techniques such as restriction fragment length polymorphism (RFLP) analysis.

3-4.2.1 Procedure:

1. The high-molecular-weight DNA strands are fractioned using restriction enzymes.

2. The DNA fragments are separated based on size by agarose gel electrophoresis.

3. The gel with the restricted fragments is then laid on a filter paper wick which serves as a connection between the membrane and the high salt buffer.

4. The nitrocellulose membrane is placed on top of the gel and a tower of filter papers is used to cover it and these are kept in place with a weight. The capillary action drives the buffer soaking through the filter paper wick, through the gel and the membrane and into the paper towels. Along with the buffer passing through the gel the DNA fragments are also carried with it into the membrane and they bind to the membrane. This causes an effective transfer of fragments (up to 15 kb in length taking around 18hours or overnight.

For DNA fragments larger than 15 kb, before blotting an acid such as diluted HCl is used to treat the gel that depurinates the DNA fragments causing breakage of DNA into smaller pieces, resulting in more efficient transfer from the gel to membrane.

Now a days blotting is also done by applying electric field. This **electro blotting** technique depends upon current and transfer buffer solution to nucleic acids onto a membrane. Following electrophoresis, a standard tank or semi-dry blotting transfer system is set up. A stack is put together in the following order from cathode to anode: sponge, three sheets of filter paper soaked in transfer buffer gel, PVDF or nitrocellulose membrane, three sheets of filter paper soaked in transfer buffer and then again sponge.

Importantly the membrane should be located between the gel and the positively-charged anode, as the current and sample will be moving in that direction. Once the stack is prepared, it is placed in the transfer system, and suitable current is applied for a specific period of time according to the materials being used.

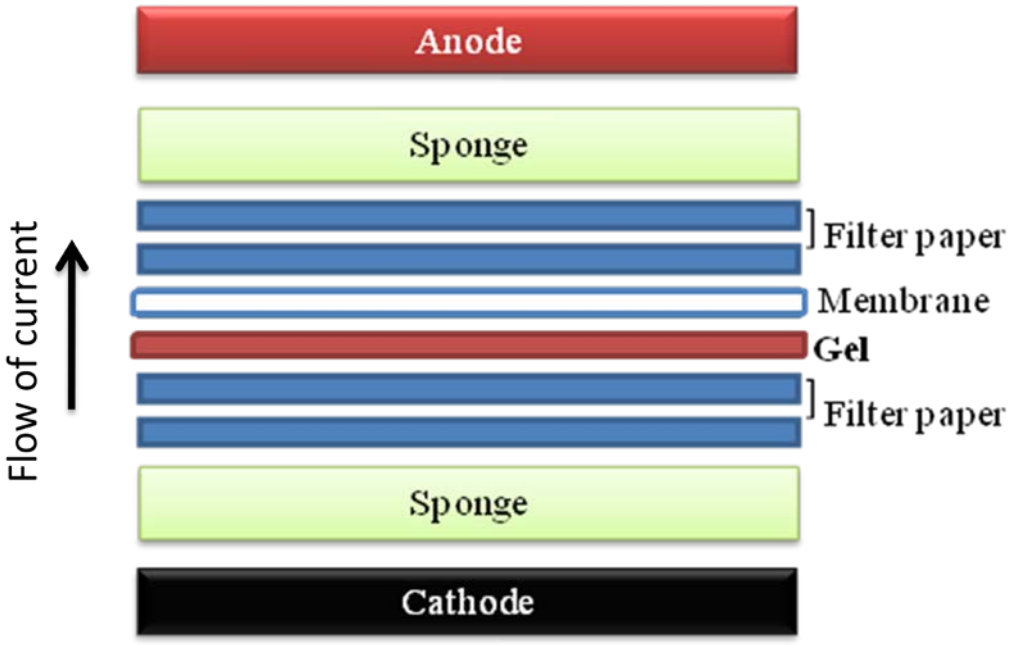


Fig 3-4.2.1: Set up for Electro-blotting system

5. For using alkaline transfer methods, the DNA gel is placed into an alkaline solution (like that of sodium hydroxide) causing denaturation of the double-stranded DNA. Denaturation in an alkaline environment enhances the binding between the negatively charged DNA and the positively charged membrane, causing separation to single DNA strands for further hybridization to the probe, alongside destroying any residual RNA that may persist in DNA. The membrane is washed with buffer to remove unbound DNA fragments.

6. The membrane which contains the transferred fragments is heated in presence or absence of vacuum at 80 °C for 2 hours or exposed to ultraviolet radiation (nylon membrane) for permanent attachment of the transferred DNA to the membrane.

7. The obtained membrane is then hybridized with a probe (a DNA fragment with a specific sequence whose presence in the target DNA is to be determined).

8. Labeling of the probe DNA is done for easy detection, usually radioactivity is incorporated or the molecule is tagged with a fluorescent or chromogenic dye. The hybridization probe may be made of RNA, instead of DNA in some cases where the target is RNA specific.

9. Washing of the excess probe from the membrane is done by using saline sodium citrate(SSC buffer) after the hybridization step and the hybridization pattern is studied on an X-ray film by autoradiography (for a radioactive or fluorescent probe), or via color development on membrane if a chromogenic detection method is employed.

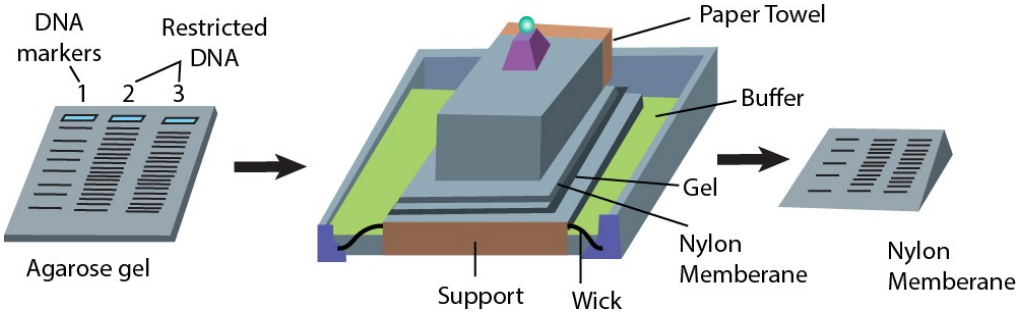


Fig 3-4.2.1: Steps of Southern Hybridization

3-4.2.2 Analysis of Southern Blot:

Hybridization of the probe to a specific DNA fragment on the membrane indicates the presence of a complementary fragment in the DNA sequence. Southern hybridization performed by digestion of genomic DNA using a restriction enzyme digestion, helps in determining the number of sequences (or gene copies) in the genome. For a probe hybridizing to a single DNA segment that has not been cut by the restriction enzyme, a single band is observed and on the other hand multiple bands will likely be observed when the hybridization occurs between the probe and several highly similar target sequence (Due to sequence duplication). Alterations in the hybridization conditions like enhancing the hybridization temperature or decreasing salt concentration, helps in altering specificity and hybridization of the probe to sequences that are less than 100% similar.

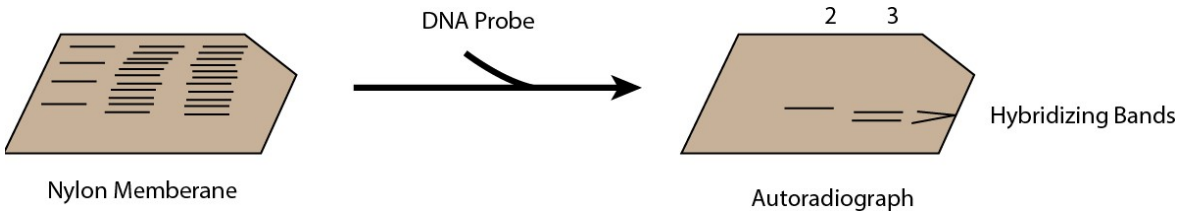


Fig 3-4.2 .2: Southern hybridization analysis

3-4.2 .3 Applications:

Southern blotting has been exploited for various applications which include:

a) Clone identification: One of the most common applications of Southern blotting is identification and cloning of a specific gene of interest. Southern blotting is carried out for identification of one or more restriction fragments that contain the gene of interest in genomic DNA.. After cloning and tentative identification of the desired recombinant by employing colony or plaque hybridization, southern blotting is further is used to confirm the clone identification and possibly to locate a shorter restriction fragment, containing the sequence of interest.

b) Restriction fragment length polymorphism Analysis: Another major application of Southern hybridization is restriction fragment length polymorphism (RFLP) mapping, which is crucial in construction of genome maps.

3-4.3 Northern hybridization:

Northern blotting was developed by James Alwine, George Stark and David Kemp (1977). Northern blotting drives its name because of its similarity to the first blotting technique, which is Southern blotting, named after the biologist Edwin Southern. The major difference is that RNA being analyzed rather than DNA in the northern blot.

Expression of a particular gene can be detected by estimating the corresponding mRNA by Northern blotting. Northern blotting is a technique where RNA fragments are separated by electrophoresis and immobilized on a paper sheet.Identification of a specific RNA is then done by hybridization using a labeled nucleic acid probe. It helps to study gene expression by detection of RNA (or isolated mRNA) in a sample.

In Northern blotting, probes formed of nucleic acids with a sequence which is complementary to the sequence or to a part of the RNA of interest. The probe can be DNA, RNA or chemically synthesized oligonucleotides of minimum 25 complementary bases to the target sequence.

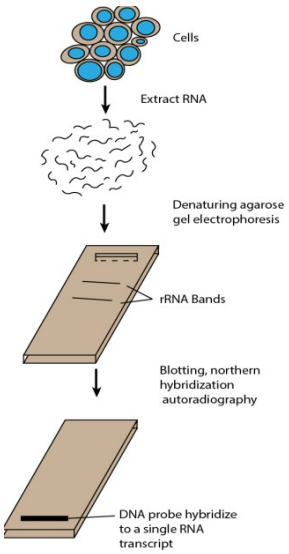


Fig 3-4.3:Steps of Northern Hybridization

3-4.3.1 Procedure:

The northern blotting involves the following steps:

1. Total RNA is extracted from a homogenized tissue sample or cells. Further eukaryotic mRNA can then be isolated by using of oligo (dT) cellulose chromatography to isolate only those RNAs by making use of a poly A tail.
2. The isolated RNA is then separated by gel electrophoresis.
3. The RNA samples separated on the basis of size are transferred to a nylon membrane employing a capillary or vacuum based system for blotting.

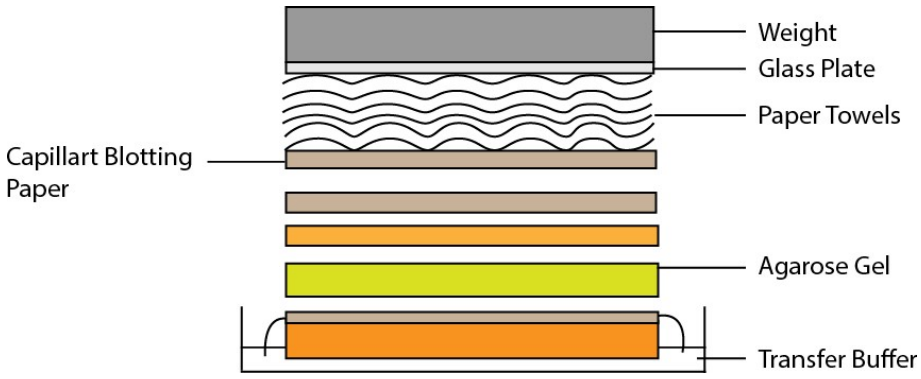


Fig 3-4.3.1: Setup for Northern blotting

4. Similar to Southern blotting, the membrane filter is revealed to a labeled DNA probe that is complementary to the gene of interest and binds.
5. The labeled filter is then subjected to autoradiography for detection.

The net amount of a specific RNA in a sample can be estimated by using Northern blot. This technique is widely used for comparing the amounts of a particular mRNA in cells under different conditions. The separation of RNA samples is often done on agarose gels containing formaldehyde as a denaturing agent as it limits the RNA to form secondary structure.

3-4.3.2 Analysis of Northern Blot:

RNA extract is electrophoresed in an agarose gel, using a denaturing electrophoresis buffer (containing formaldehyde) to ensure that the RNAs do not form inter- or intra-molecular base pairs, as base pairing would affect the rate at which the molecules migrate through the gel. After electrophoresis, the gel is blotted onto a nylon or nitrocellulose membrane, and hybridized with a labeled probe. If the probe is a cloned gene, the band that appears in the autoradiograph is the transcript of that gene. The size of the transcript can be determined from its position within the gel, and if RNA from different tissues is run in different lanes of the gel, then the possibility of differentially expressed gene can be examined.

3-4.3.3 Applications:

Northern blotting helps in studying gene expression pattern of various tissues, organs, developmental stages, pathogen infection, and also over the course of treatment. It has been employed to study overexpression of oncogenes and down-regulation of tumor-suppressor genes in cancerous cells on comparison with healthy tissue, and also for gene expression of immune-rejection of transplanted organ.

The examination of the patterns of gene expressions obtained under given conditions can help determine the function of that gene.

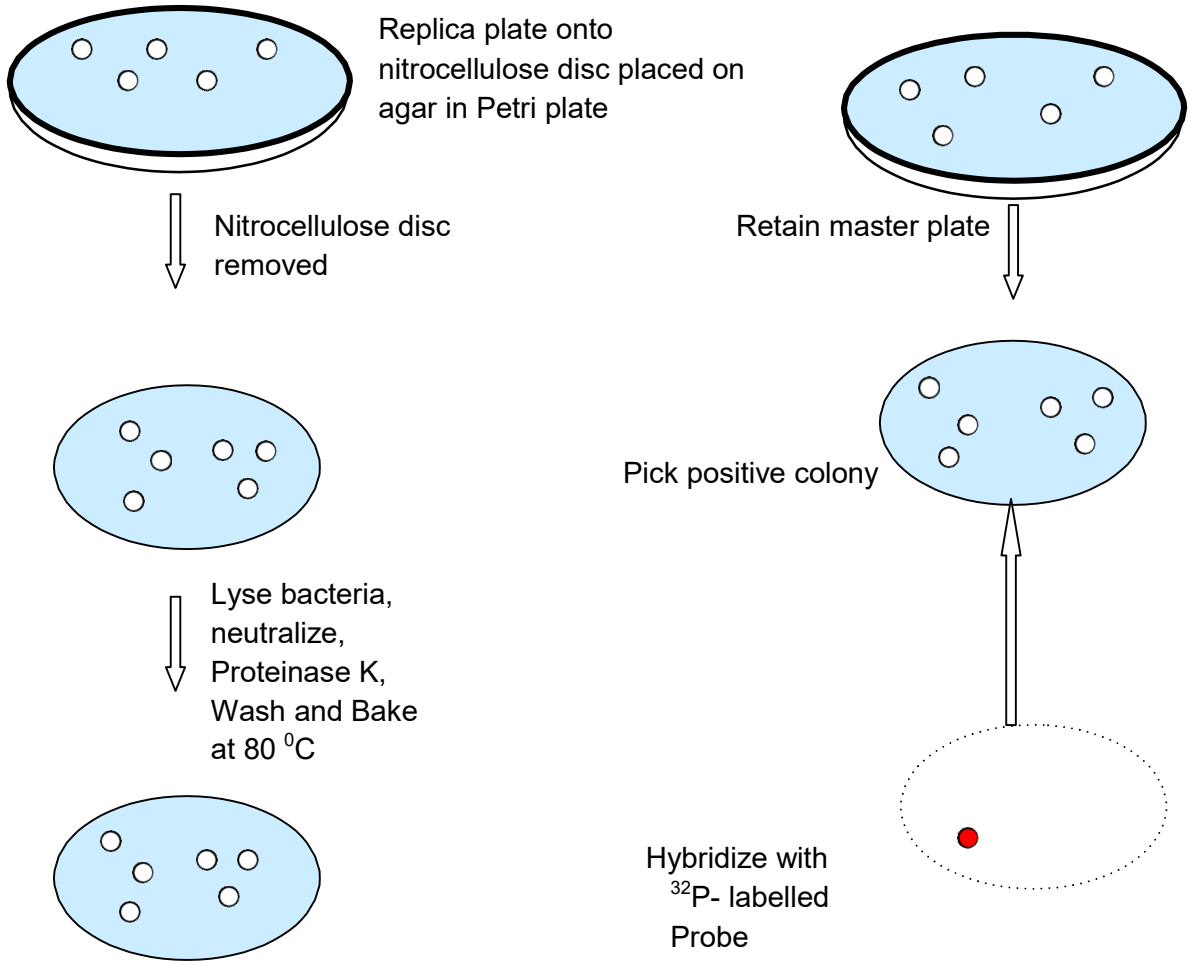
Northern blotting is also used for the analysis of alternate spliced products of same gene or repetitive sequence motif by investigating the various sized RNA of the gene. This is done when only probe type with variation in one location is used to bind to the target RNA molecule.

Variations in size of a gene product may also help to identify deletions or errors in transcript processing, by altering the probe target that can be used along the known sequence and make it possible to determine the missing region of the RNA.

3-4.4 Colony Hybridization:

It is a rapid method of isolating a colony containing a plasmid harboring a particular sequence or a gene from a mixed population. The colonies to be screened are first replica-plated on to a nitrocellulose filter disc that has been placed on the surface of an agar plate prior to inoculation. Master plate is retained for reference set of colonies. The filter bearing the colonies is removed and treated with alkali so that the bacterial colonies are lysed and the DNA they contain is denatured. The filter is then treated with proteinase K to remove protein and leave denatured DNA bound to the nitrocellulose. The DNA is fixed firmly by baking the filter at 80°C. A labeled probe is hybridized to this DNA which is monitored by autoradiography. A colony whose DNA print gives a positive auto radiographic result on X-ray film can then be picked from the reference plate.

Colony hybridization can be used to screen plasmid or cosmid based libraries.



DNA Print

Autoradiograph

Fig 3-4.4: Colony hybridization

3-4.5 *In Situ* Hybridization (ISH)

It is a technique that employs a labeled complementary nucleotide strand (i.e. probe) for localizing specific DNA or RNA sequence targets within fixed tissues and cells (i.e *in situ*). Probes used for hybridization can be double-stranded DNA probes, single-stranded DNA probes, RNA probes, synthetic oligonucleotides. There are two ways available to detect DNA or RNA targets

Chromogenic (**CISH**) in situ hybridization and Fluorescence in situ hybridization (**FISH**).

3-4.5.1 Chromogenic in situ hybridization (CISH)

It uses the labeling reactions involving alkaline phosphatase or peroxidase reactions to visualize the sample using bright-field microscopy. It is primarily used in molecular pathology diagnostics. CISH can also be employed for samples like fixed cells or tissues, blood or bone marrow smears and metaphase chromosome spreads.

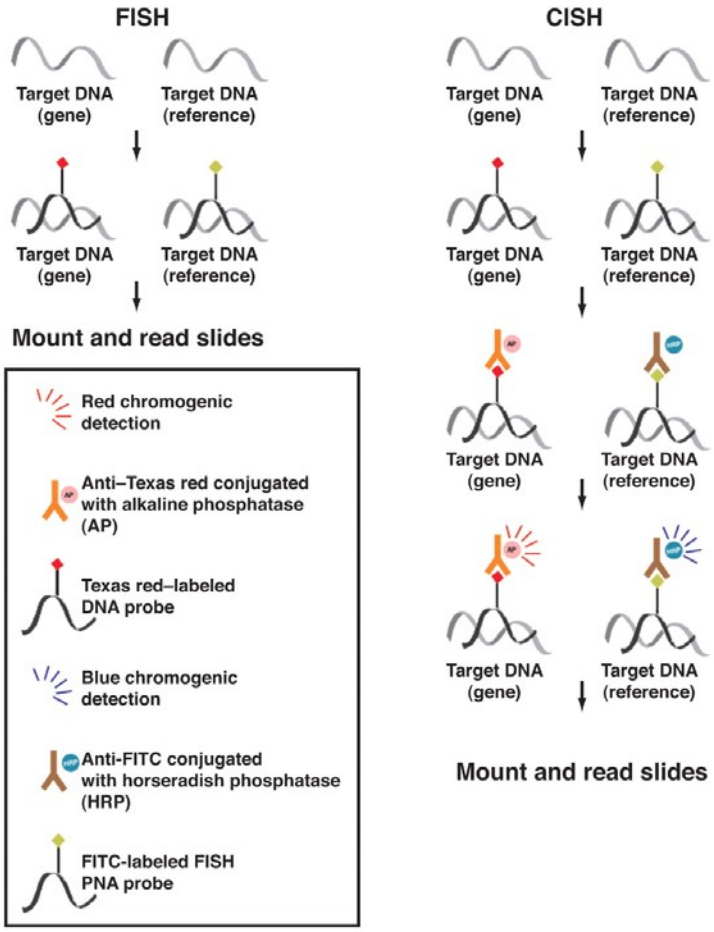


Fig.3-4.5.1 Use of dual-color Chromogenic in situ hybridization (CISH) in combination with fluorescence in situ hybridization (FISH) probes. FITC, fluorescein isothiocyanate; PNA, peptide nucleic acid.

(Obtained from http://ajcp.ascpjournals.org/content/133/2/205/F1.expansion.html)

3-4.5.2 Fluorescence in situ hybridization (FISH)

FISH is a cytogenetic technique that uses fluorescent probes that bind to complementary targets and sample is visualized using epi-fluorescence or confocal microscopy. Using differently labeled probes, we can visualize several targets in a single sample. It is used for spatial-temporal patterns of gene expression and resolving genetic elements in chromosomal preparations.

Cells or tissues to be analyzed are fixed and permeabilized with Proteinase K to allow target accessibility. Probe is constructed and tagged using non-radioactive labels like biotin, digoxigenin or fluorescent dye (FISH). Probe must be large enough to hybridize specifically with its target. Probe is applied to fixed sample and incubated to several hours to allow hybridization. Washing is done to remove non-specific or unbound probe removal. Results are then visualized using either bright-field or confocal microscopy.

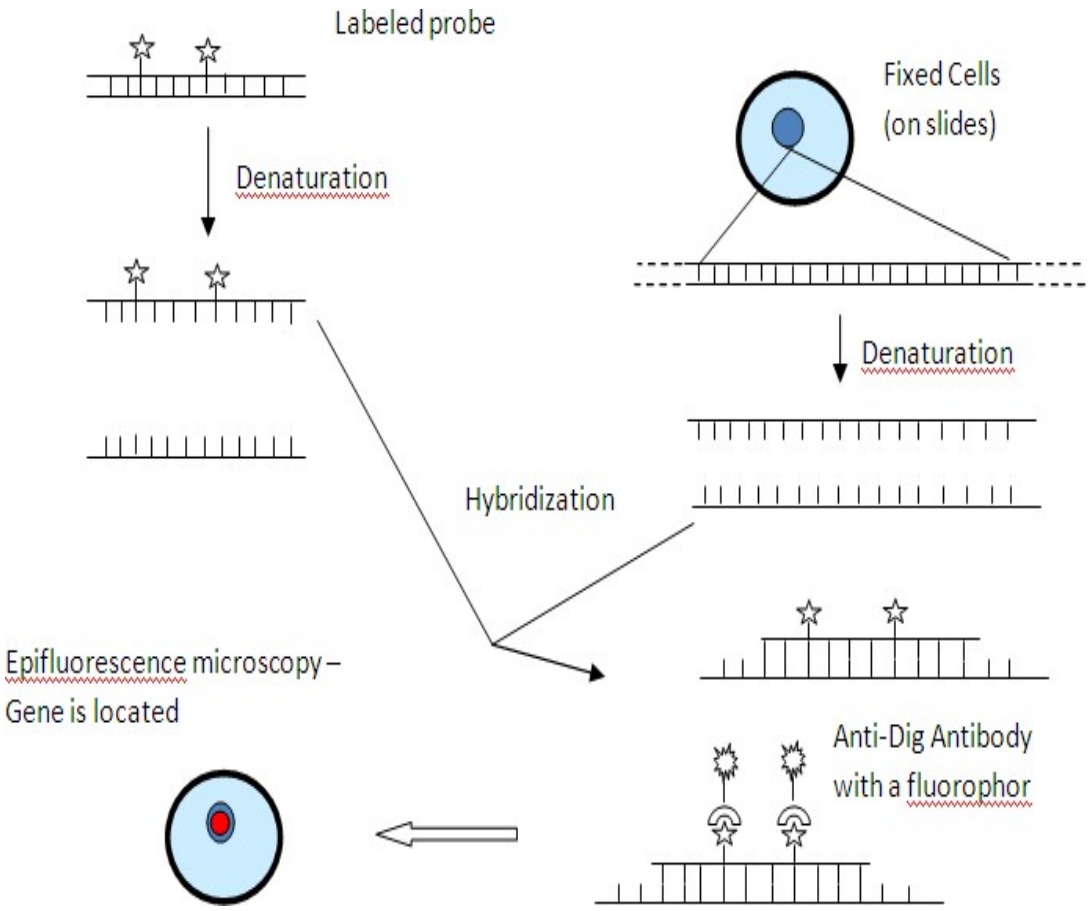


Fig3-4.5.2: Fluorescent in-situ hybridization

3-4.6 Dot Blot and Slot Blot Hybridization

These two techniques represents the simplification of Southern and Western blots saving the time involved in procedures of chromatography, electrophoresis, restriction digestion and blotting of DNA or proteins from the gel to membrane. Here nucleic acid mixture is directly applied (blotted) on to the nylon or nitrocellulose membrane where hybridization between probe and target takes place, denatured to single-stranded form and baked at 80°C to bind DNA target to membrane. In dot-blot, target is blotted as circular blots whereas in slot-blots, it is in the form of rectangular blots. Due to this, slot-blot offers greater precision in observing different hybridization signals. After blotting, membrane is allowed to dry and non-specific sites are blocked by soaking in blocking buffer containing BSA. It is then followed by hybridization of labeled probe for detection of specific sequences or gene.

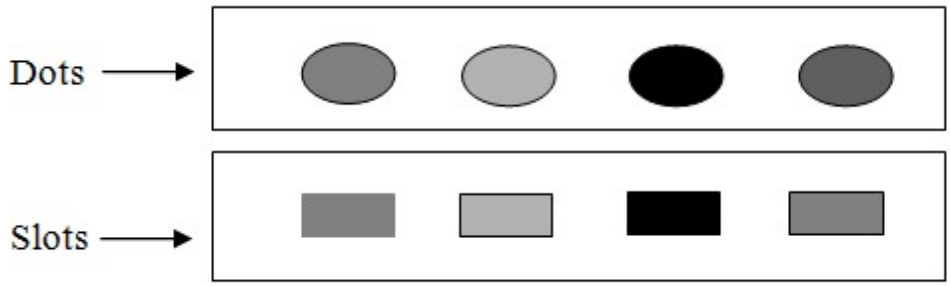


Fig 3-4.6 Dots and slots in dot and slot blot hybridization

These procedures can only detect presence and absence of particular sequence or gene. It cannot distinguish between two molecules of different sizes as they appear as single dot on membrane. It also has application in detecting alleles that differ in single nucleotide with the help of allele-specific oligonucleotides.

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