RECOMBINANT DNA TECHNOLOGY

II M.Sc., MICROBIOLOGY
SEMESTER III

UNIT III (SCREENING AND SELECTION OF RECOMBINANTS)

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SCREENING AND SELECTION FOR RECOMBINANTS

The art of cloning is to find the one particular transformed cell/recombinant cell that contains the cloning vector with the
gene of interest (referred to as a recombinant cell).

It is thus most important to be able to select recombinant cells from transformed cells (containing the vector without insert DNA).

IDENTIFICATION OF RECOMBINANTS:

After the introduction of rDNA into suitable host cells, it is essential to identify those cells which have received the rDNA molecules. This process is called **screening or selection.**

Generally, the **selection methods are based on the expression or non-expression of certain traits such as antibiotic resistance, expression of an enzyme such as β- galactosidase or protein such as GFP (Green Fluorescent Protein) and dependence or independence of a nutritional requirement such as the amino acid leucine.**

Direct selection, which means that the cloning experiment is designed in such a way that the clones obtained are the clones containing the required gene. Almost invariably, selection occurs at the plating-out stage. Direct selection is the method of choice, as it is quick and usually unambiguous. However, it is not applicable to all genes.

Identification of the clone from a gene library, which entails an initial “shotgun” cloning experiment, to produce

I) DIRECT SELECTION OF RECOMBINANTS:

Two examples will be discussed:

(i) direct antibiotic resistance screening, and
(ii) blue-white color screening.

II) INDIRECT SELECTION OF RECOMBINANTS:

(i) Screening by Nucleic acid hybridization
(ii) Screening by Colony hybridization
(iii) Screening by Immunological assay
(iv) Screening by Protein/Enzyme activity

1.) Direct antibiotic resistance screening

Most cloning vectors are designed so that the insertion of a DNA fragment into the vector **destroys the integrity of one of the genes present on the molecule** (usually an antibiotic resistance gene).

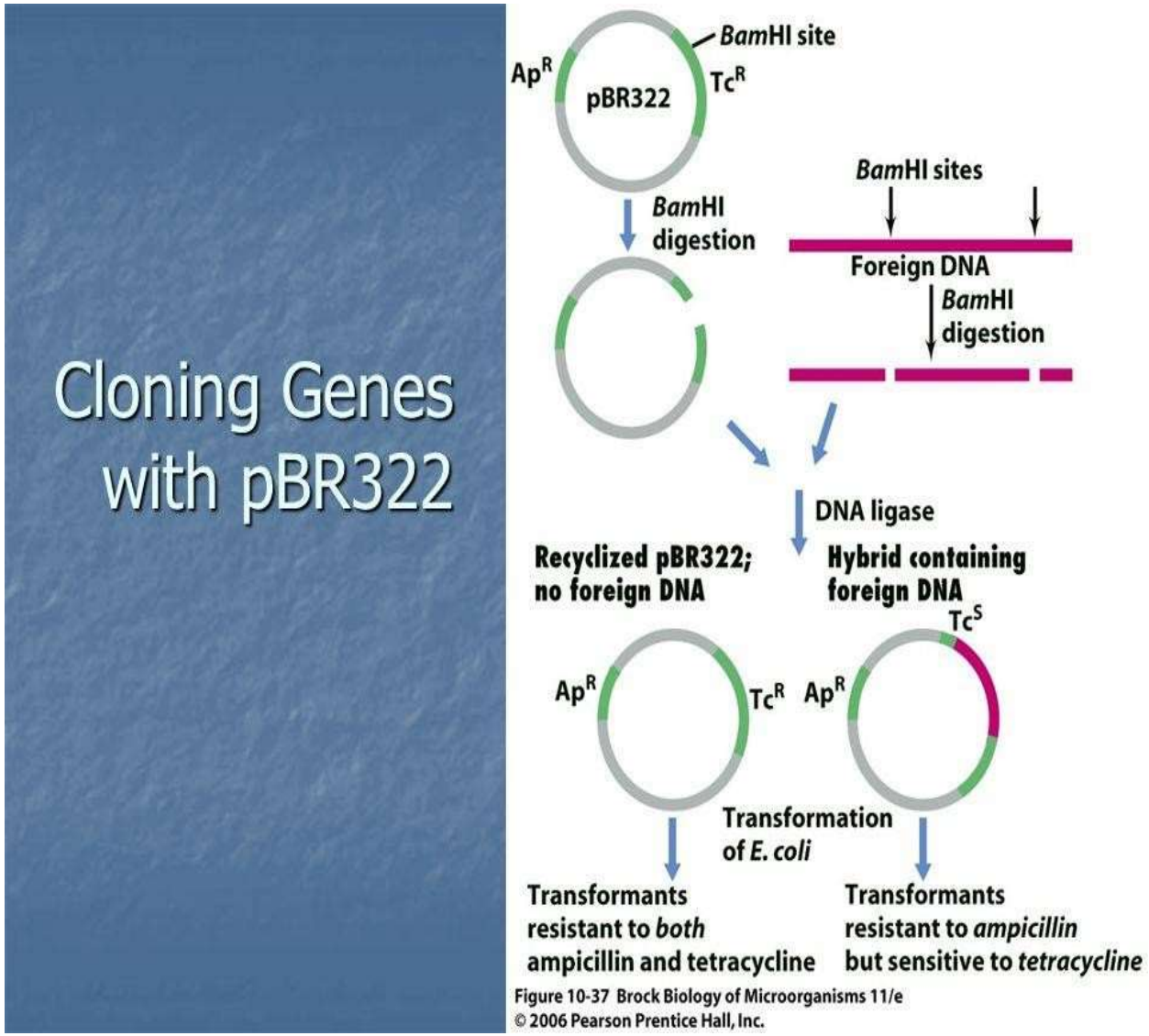
INSERTIONAL INACTIVATION METHOD:

In this approach, one of the genetic trait is disrupted by inserting foreign DNA. **Antibiotic resistance genes act as a good insertion inactivation system.**

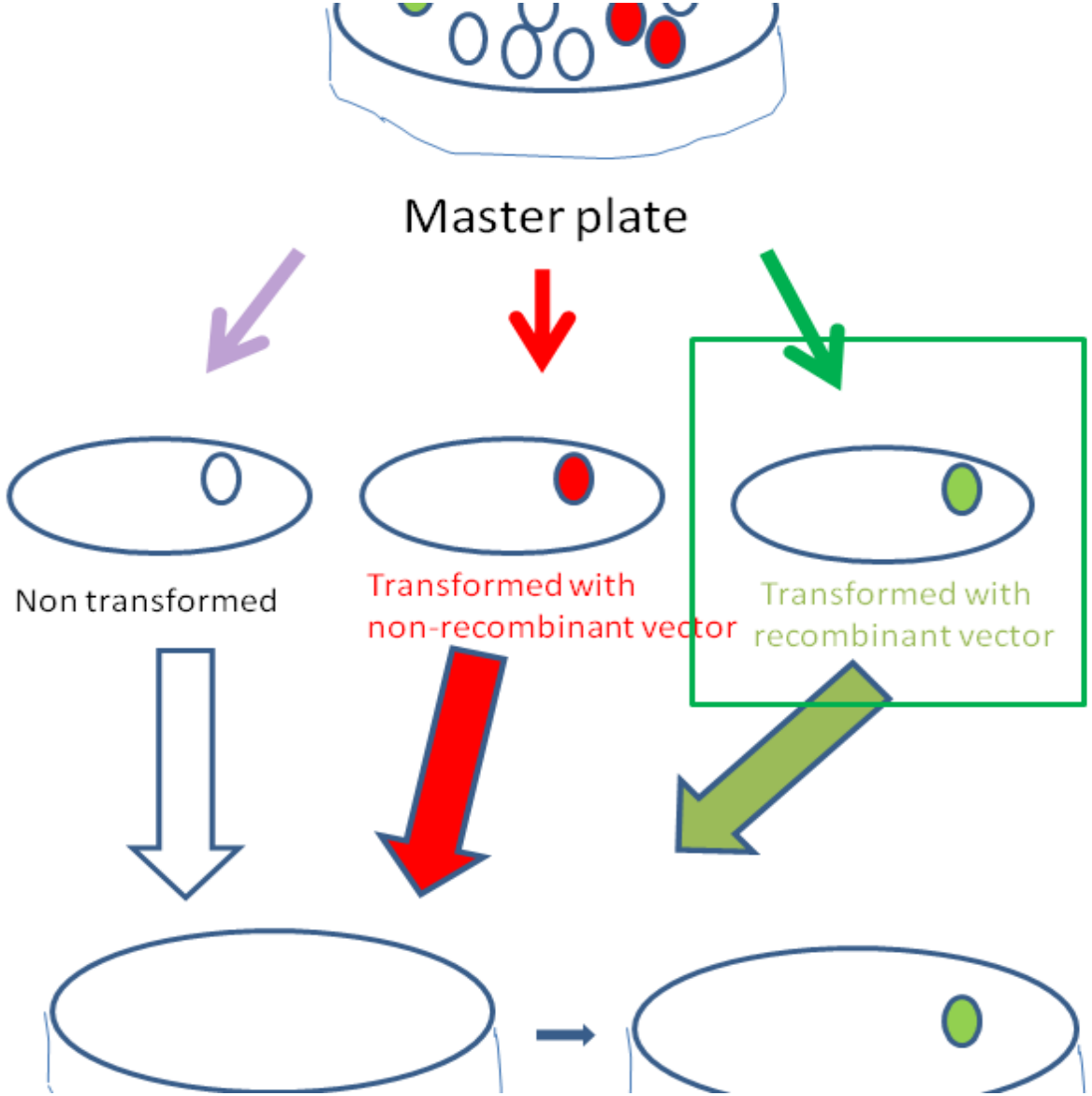
Plasmid pBR322 contains two antibiotic resistance genes, one for ampicillin (amp^{r}gene), and the other for tetracycline (tet^r gene).

If the target DNA is inserted into tet^r gene using Bam HI, the property of resistance to tetracycline will be lost. Such **recombinants would be tet^{s}sensitive.**

When such recombinants (containing target DNA in tet^r gene) are grown into medium containing tetracycline, they will not grow because their tet^r gene has been inactivated. But they are resistant to ampicillin because amp^r gene is functional.



On the other hand, the self-ligated recombinants will show resistance to ampicillin and tetracycline. Therefore, they will grow on medium containing both the antibiotics.



Selection of recombinants

1) Non-transformed:

Cannot grow on ampicillin or tetracycline medium

2) Transformed:

Only transformed colonies can grow in ampicillin or tetracycline containing medium.

a) **Transformed with non recombinant** or unaltered vector, can grow in both ampicillin and tetracycline containing medium

b) **Transformed with recombinant vector** carrying our gene of interest. Transformed recombinants can grow only in ampicillin medium and cannot grow on tetracycline

Blue white selection-

2. Visual Screening :Blue-white colour screening

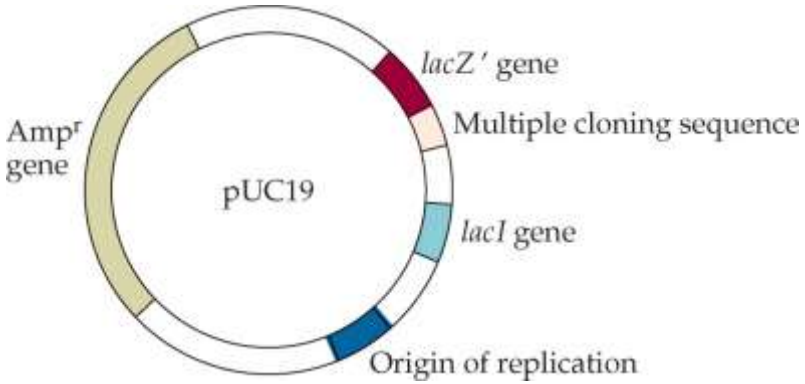
A more sophisticated procedure for screening for the presence of recombinant plasmids, which can be carried out on a single transformation plate, is called blue-white screening.

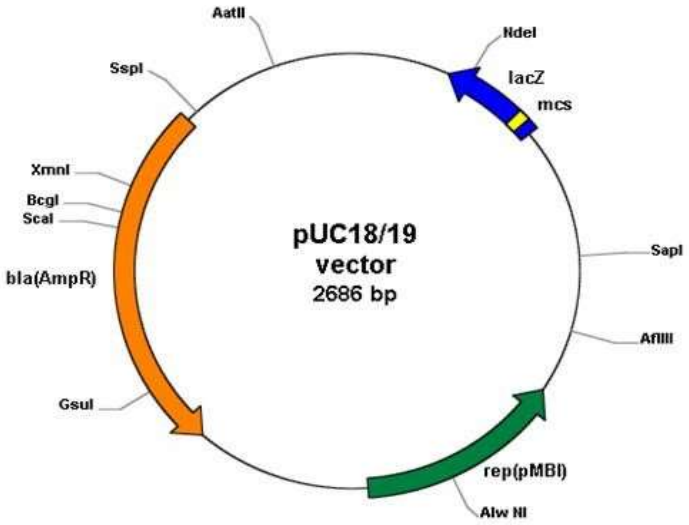
This method also involves the insertional inactivation of a gene and uses the production of a blue compound as an indicator. The gene is *lacZ*, which encodes the enzyme ß-galactosidase, and is under the control of the *lac* promoter.

If the host *E. coli* strain is expressing the lac repressor, expression of a *lacZ* gene on the vector may be induced using IPTG (isopropyl- ß-D-thiogalactopyranoside), and the expressed enzyme can utilize the synthetic substrate X-gal (5-bromo-4-chloro-3-indolyl- ß-D-galactopyranoside) to yield a blue product.

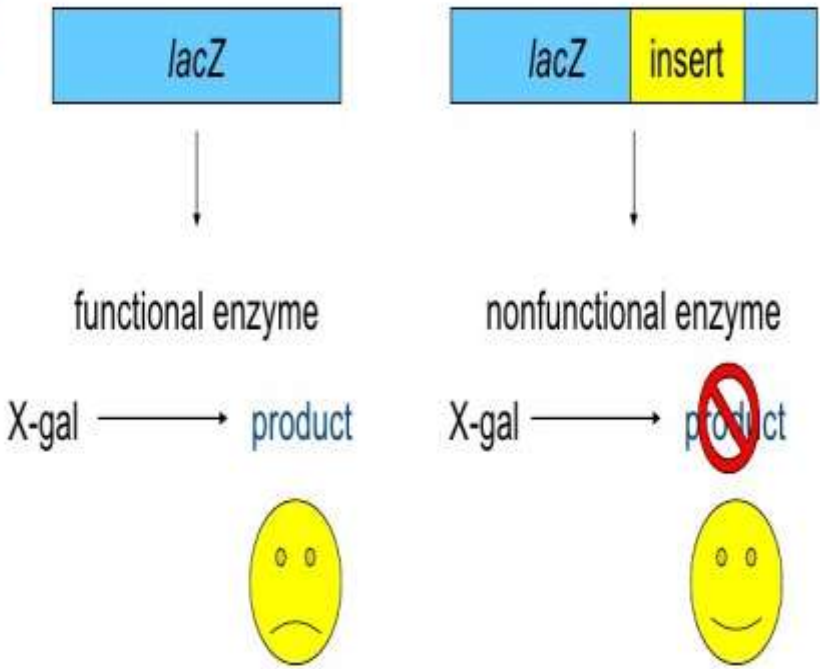
Cloning of insert DNA using β - galactosidase: as reporter gene

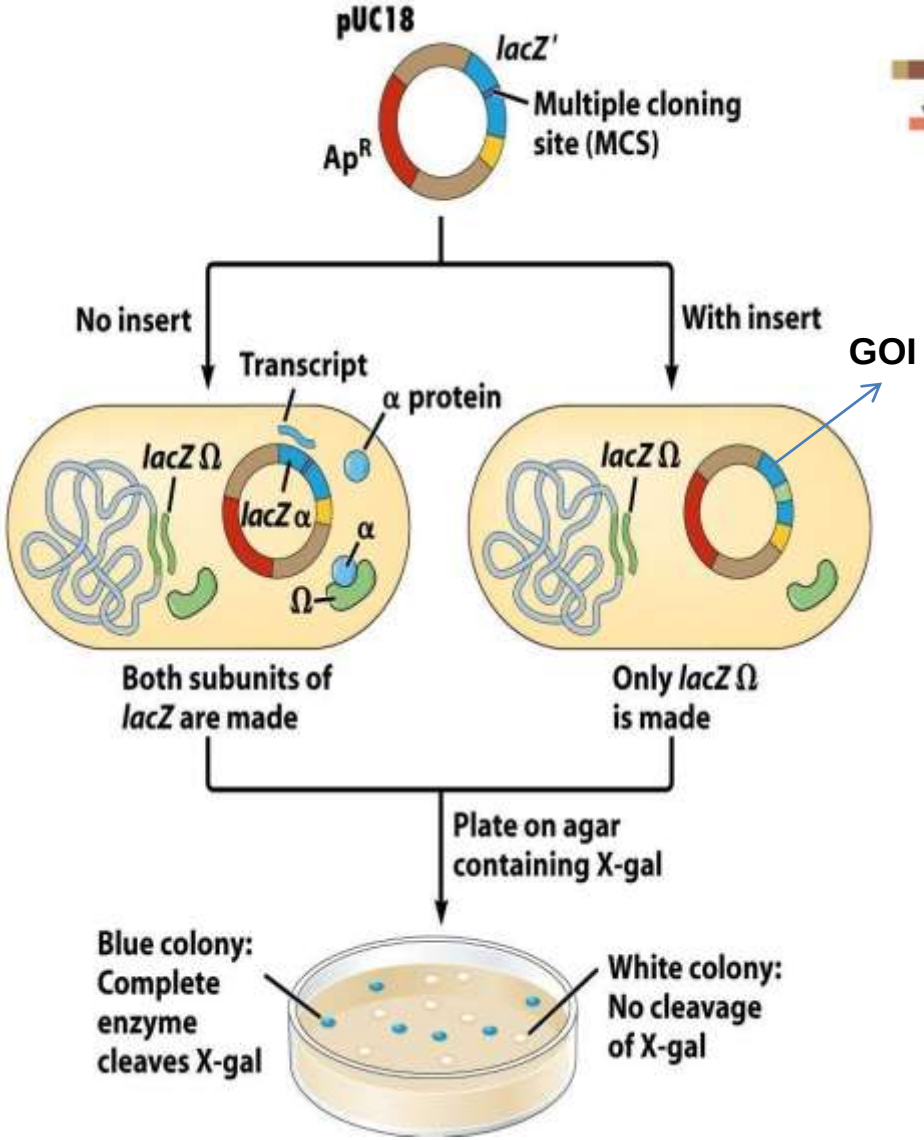
1. Multiple cloning site is inserted into the gene lacZ' coding for the alpha peptide of enzyme β-galactosidase. (An MCS is a short DNA sequence consisting of restriction sites for many different restriction endonucleases).
2. Insertion of the MCS into the lacZ' fragment does not affect the ability of the α-peptide, while cloning DNA fragments into the MCS does.
3. Clones with foreign DNA in the MCS disrupt the ability of the cells to make β-galactosidase.
4. Plate on media with a Substrate X-gal (β-galactosidase indicator) and clones with intact β-galactosidase enzyme indicate that cells containing empty vector without insert, will produce blue colonies. Colorless (desirable) colonies indicates that cells contain the plasmid with foreign DNA
5. Therefore, recombinants can be detected by blue/white screening on growth medium containing X gal in presence of IPTG as an inducer.

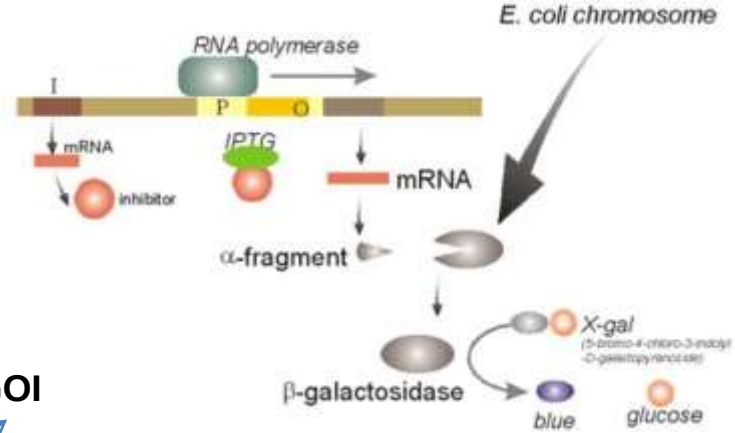




Blue/White Color Screening







β-galactosidase enzyme + chromogenic substrate (X-gal)

Blue color colonies

Cloning in pUC18/19:

II) Indirect methods:

Identification of the clone from a gene library:

Many different techniques are available for screening a library.

The most important approaches involve

screening by nucleic acid hybridization and screening by Colony hybridization

screening by functional analysis- Immunological assay - Protein Assay

SELECTION OF TRANSFORMANTS – Marker Genes- SELECTABLE MARKER GENES

Marker genes are present on vector into which the DNA of interest has been cloned. These genes protect the organism from a selective agent that would normally kill it. All cells that do not contain the foreign DNA are killed in the presence of selective agent and only the desired ones are left behind. Marker genes are used as tools in

- molecular biology (transformation studies)
- metagenomics (study direct environmental sample)
- phylogenetic analysis (genetic lineage)

They are used to determine whether a particular piece of DNA or gene of interest is successfully inserted into the target DNA.

It is also used to determine orthologous gene groups.

It is present next to the transgene.

It is of two types: screenable markers and selectable markers.

DIFFERENCE BETWEEN SELECTABLE MARKER AND REPORTER GENE

Selectable Marker	Reporter Gene
Definition	
Selectable markers are sequences that can be used to distinguish between transformed and non-transformed genes.	Reporter genes are test sequences whose expression can be quantified.
Function	
They act as markers for transformed genes by providing resistance against toxic substances.	The amount of protein a reporter gene produces can be quantified to assess the transformation of a gene.
Examples	
Antibiotic resistance genes, herbicide resistance genes and antimetabolite marker genes.	Green fluorescent protein, luciferase, octopine synthase.

Selectable Markers

Selectable markers will protect the organisms against a selective agent which kills or prevents its growth.

Since the frequency of transformation is less, the use of these selectable marker enables researchers to make quick decisions. The most commonly used selective agents are the antibiotics selection. Chloroplast killing (**Eg. Streptomycin, neomycin**) and mitochondria killing (**Eg. Penicillin**) antibiotics are available. There are different categories of selectable marker genes are:

- **Antibiotic resistance gene markers-** bacterial origin mostly from E.coli
  - i. **Neomycin phosphotransferase-** NPT II is the most widely used marker in plant transformation. It provides resistance to kanamycin.
  - ii. **Hygromycin phosphotransferase-** hpt gene is used as marker in plant transformation and more powerful than kanamycin. It kills non-transformed cells by blocking protein synthesis.
- **Herbicide resistance gene markers**
  - i. **Bar gene-** is cloned from *Streptomyces hygroscopicus.* Bialaphos (secondary metabolite) contains phosphinothricin, which prevents glutamine synthetase thereby preventing nitrogen metabolism. Accumulation of excess ammonium in plants will result in disruption of primary metabolism. The bar gene has modifying enzyme called phosphinothricin acetyl transferase to prevent phosphinothricin activity.
  - ii. **Bromoxylin nitrilase (bxn)-** it can alter the structure of herbicide bromoxynil thereby preventing the damage to photosynthetic machinery. It can be derived from ***Klebsiella ozaenue***.

Screenable Markers

Screenable markers are also referred to as reporter genes or scorable markers.

Often accompanied with colour change or visible phenotypic changes in the tissues of transformed organisms.

They do not affect the growth of the cells.

Preferred when a large group of cells have to be screened.

They are used for quantitative assays.

Types of Screenable Markers

Green Fluorescent Protein (GFP)

○The gene is derived from jelly fish (***Aequorea victoria***). This protein has 238 amino acids of which amino acids serine, tyrosine and glycine imparts the chromophore. It makes the light glow green under UV light (bioluminescent). It is used to measure gene expression.

GUS Assay (β- glucuronidase)

○Most widely used reporter gene in plant transformation. GUS gene is analyzed by immersing the tissues in solution containing the substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid).

○**Opine synthase.** These genes are present in Ti plasmid and both octopine and nopaline are considered as reporters.

Luciferase

○Fire fly based luciferase gene, a non-toxic reporter. The samples placed in luciferin solution in presence of ATP, oxidation emits light captured in X-ray films.

Blue/ white screening

○It is efficiently used in identification of recombinant bacteria. The lacZ gene makes cells turn blue in X-gal media which is based on the activity of β- galactosidase activity.

Chloramphenicol Acetyl Transferase (CAT)

○First reported bacterial reporter gene. This method is very sensitive and requires radioactive assay with substrate labeling.

Reporter Genes

An alternative to selectable marker gene is a reporter gene which helps in distinguishing between wanted and unwanted cells.

Ideally a reporter gene should have following properties:

i. Its product should be easy to assay
ii. There should be little or no endogenous activity for this gene
iii. It should be non-toxic
iv. It should tolerate N-terminal fusions

Commonly used reporter genes are:

- **Chloramphenicol acetyltransferase**
- **β-galactosidase**
- **Nopaline synthase**
- **Octopine synthase**

Sequence-dependent screening

Sequence-dependent screening can be achieved by exploiting the homologous sequence of the desired gene. Sufficient information about the sequence of interest is required to make suitable probes and primers. This is done by following two methods:

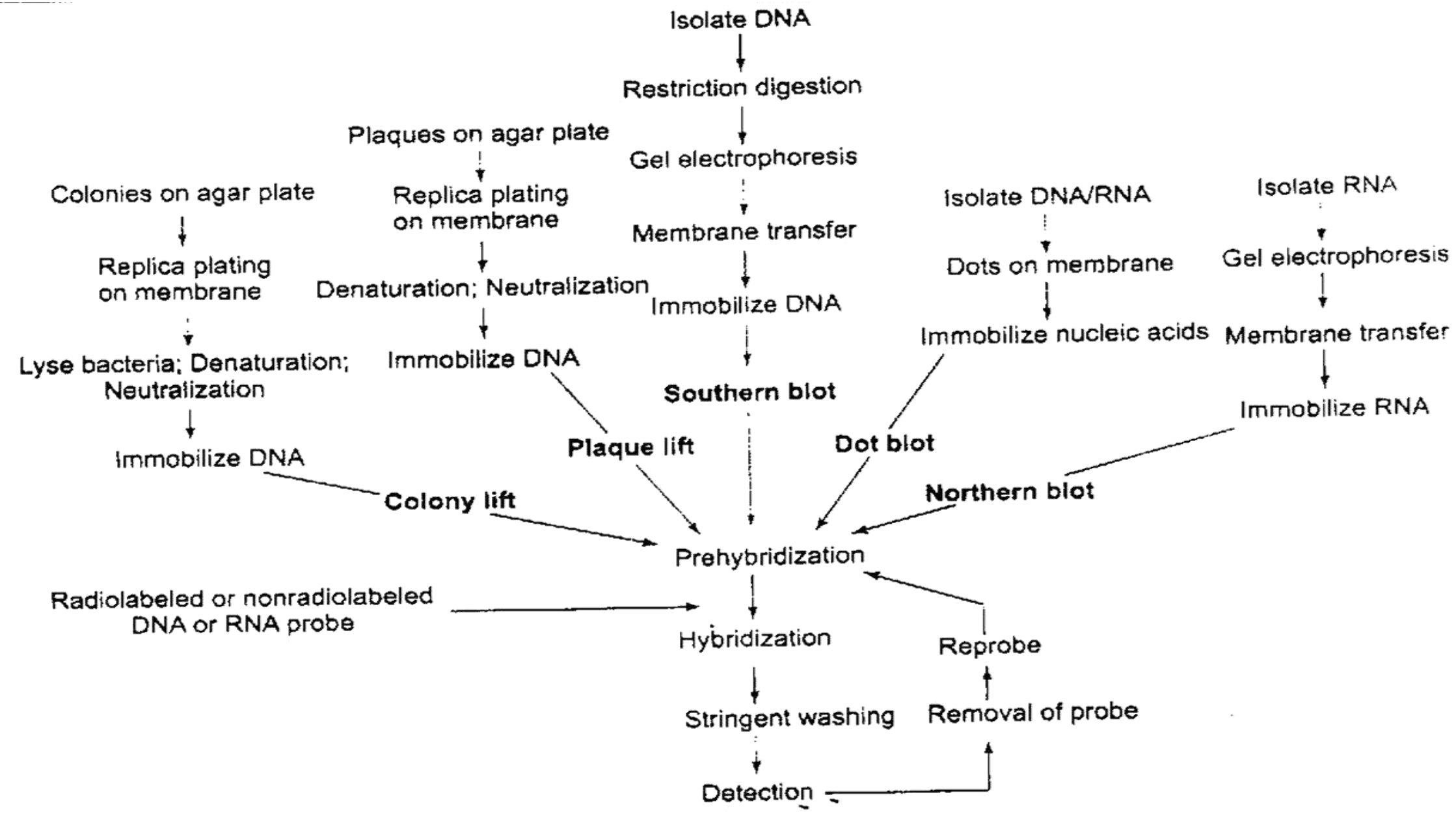
- **Nucleic acid hybridization technique**

The clones (colonies or plaques) of a library are transferred to a membrane made up of nitrocellulose or nylon. The colonies on the membrane are gently lysed and DNA coming out of the cells is immobilized on the membrane. The desired clone is selected by hybridization of the immobilized DNA with a labeled DNA probe.

- **PCR technique**

The clones are screened by colony PCR method by using primers which are designed based on the available sequence information.

An Overview of Nucleic Acid Hybridization



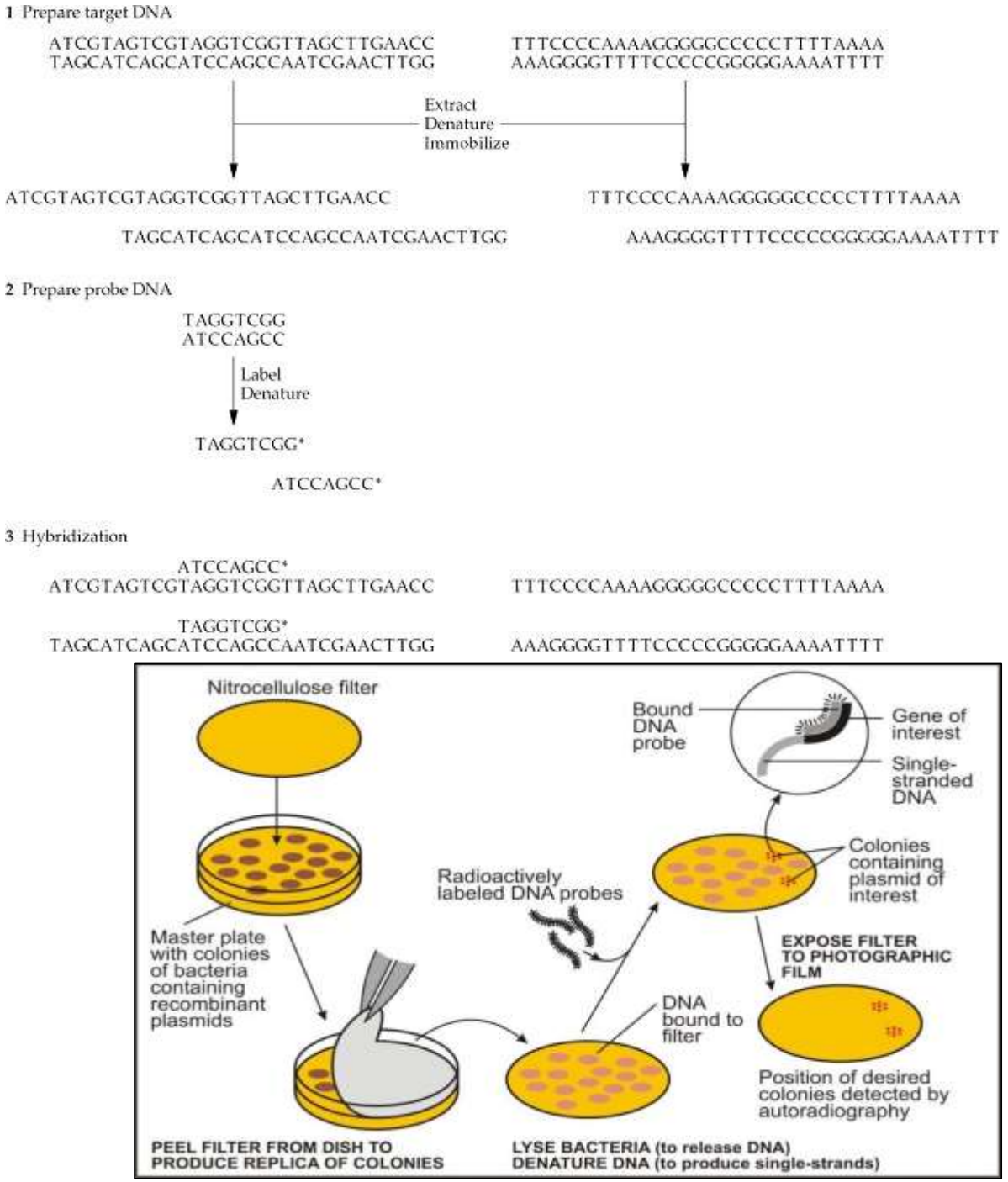
Screening by Nucleic acid hybridization

1. The first step of a hybridization screening experiment involves DNA from the clones are isolated and separated on the agarose gel electrophoresis. The transfer of the DNA from the gel to a nylon or nitrocellulose membrane is carried out.

2. The DNA is denatured with alkali to produce single strands that are bounded to the membrane by heat treatment or UV irradiation.

3. The membrane is then immersed in a solution containing a nucleic acid probe (usually radioactively labeled) and incubated to allow the probe to hybridize to its complementary sequence.

4. After hybridization, the membrane is washed to remove unhybridized probe, and regions where the probe has hybridized are visualized with autoradiography.



Hybridization Procedures

In these techniques nucleic acid hybridization is used to detect and quantify specific DNAs/RNAs. The DNAs/RNAs to be probed are immobilized on a membrane and hybridized with a labeled DNA/RNA probe. The probe hybridizes to its complementary sequence and the hybridization product is detected with a detection method. A nucleic acid hybridization technique has the following steps:

- **Immobilization of the target DNA/RNA**

The target nucleic acid to be probed is isolated and immobilized on a nitrocellulose or nylon membrane. Nitrocellulose membranes have low capacity to bind nucleic acids and cannot be used for repeated hybridizations since these become brittle during the baking step of immobilization.

These limitations of the nitrocellulose membranes have been overcome with the introduction of various types of nylon membranes. The nucleic acids are either blotted to the membrane from a gel or spotted directly on the membrane.

If the nucleic acid is double stranded DNA, it is denatured just before blotting/spotting. The bacterial colonies can be transferred to the membranes and lysed to liberate DNA which is immobilized subsequently to the membrane. The nucleic acid blotted to the membrane is immobilized by baking in a vacuum/microwave oven or cross-linking by UV irradiation, depending upon the membrane used.

The transfer of DNA from an agarose gel to a membrane is called as Southern blotting after the name of the scientist Edwin M. Southern who introduced this technique in 1975. The RNA transfer from the gel to the membrane has been given the name northern blotting.

Hybridization Procedures

•Preparation of the labeled probes

A labeled DNA or RNA molecule having sequence complementary to a target nucleic acid molecule is used as a probe. A probe may have complete or partial homology to the nucleic acid molecule to be probed and it could be end-labeled or continuously labeled.

An end-labelled probe is made by adding a label at the 5′ or 3′-end. A continuously labeled probe is produced by nick translation, random priming, PCR, *in vitro* transcription or cross-linking with a label. A label on DNA or RNA could be radioisotopic or non-isotopic. The radioisotopic labels used are ^{32}P, ^{3}H and ^{35}S. Among these ^{32}P is the most commonly used radioisotopic label.

A nucleoside triphosphate precursor, labeled at the α or γ position, is used to label nucleic acids. A commonly used technique for radiolabelling of DNA is the nick translation technique which was discovered by Paul Berg and his coworkers in 1977.

In this technique random nicks are introduced in a double stranded DNA molecule by DNase I enzyme and the normal nucleotides near the nick are replaced by radio-labeled nucleotides by the joint action of 5′- 3′ exonuclease and 5′- 3′ polymerase activities of *E. coli* DNA polymerase I enzyme in the presence of one radiolabeled dNTP and four normal dNTPs.

The commonly used non-isotopic labels for making nucleic acid probes are biotin, digoxigenin and fluorescein. The radioisotopic probes are highly sensitive and compatible with several labeling techniques, however, these probes suffer from the disadvantages of being potential health hazards and having short half life of the isotope.

Moreover these are not suitable for repeated probing of the target nucleic acid molecules. Non-isotopic labels are quite safe to use and can be stored for long periods.

Hybridization of the probe with the target nucleic acid

The membrane on which target nucleic acid is immobilized is placed in a hybridization container. A pre-hybridization buffer is added to the hybridization container to prevent non-specific binding of the probe to the membrane.

After treatment for 1.5 hrs to 12 hrs, the pre-hybridization buffer is removed and the hybridization solution (pre-hybridization buffer containing the denatured probe) is added to the hybridization container.

The main components of the pre-hybridization/hybridization buffer are: rate enhancer, detergent, blocking agent, heterologous DNA and denaturant. The hybridization container is placed on a roller. After overnight incubation, the hybridization solution is discarded into a proper liquid waste container and the membrane is given a series of washes with a suitable washing solution.

Detection of the probe

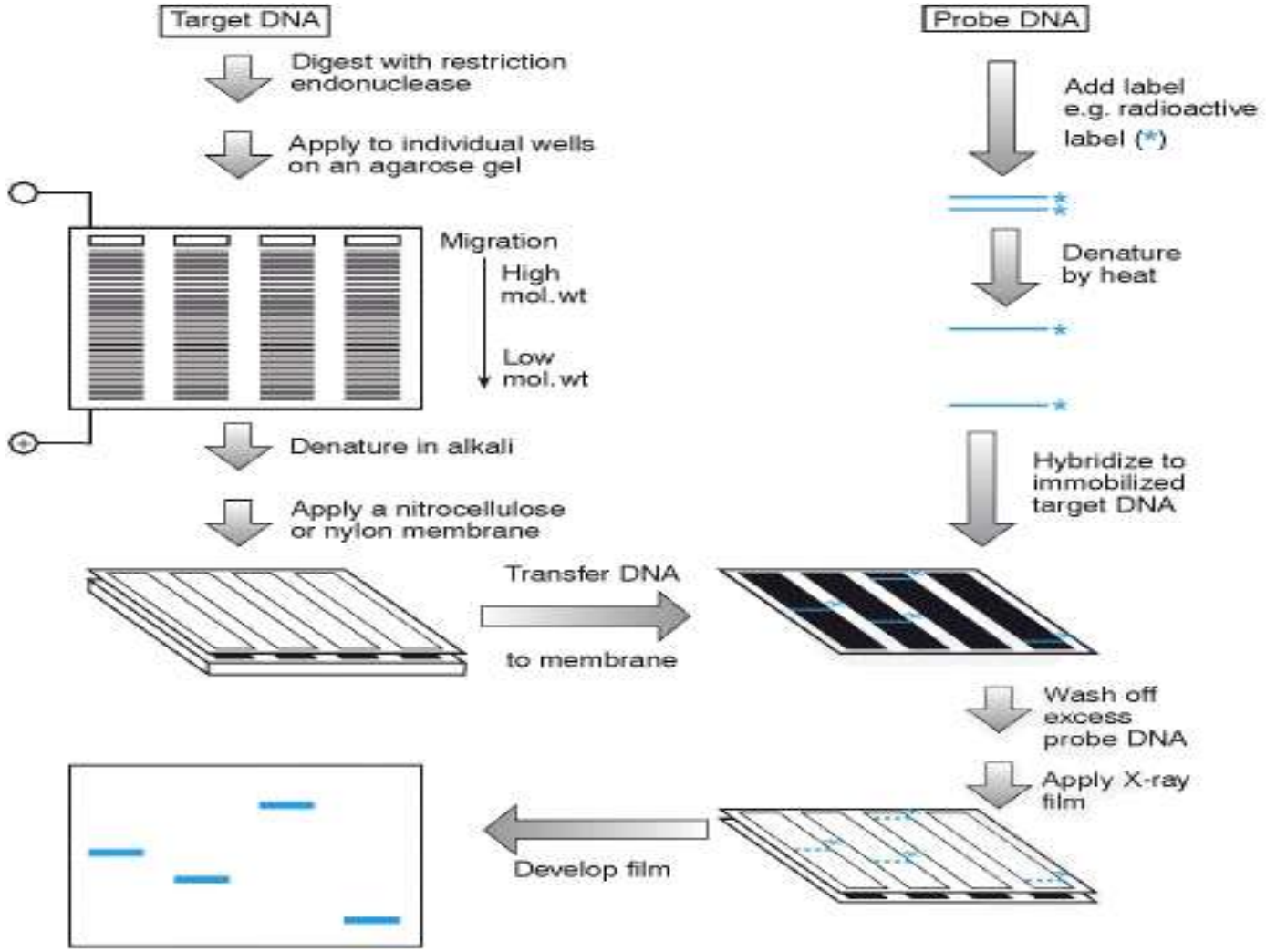
The probe on the washed membrane is detected by an appropriate method. The selection of the method is made based on the type of probe used. The radioisotopic probes are detected by autoradiography.

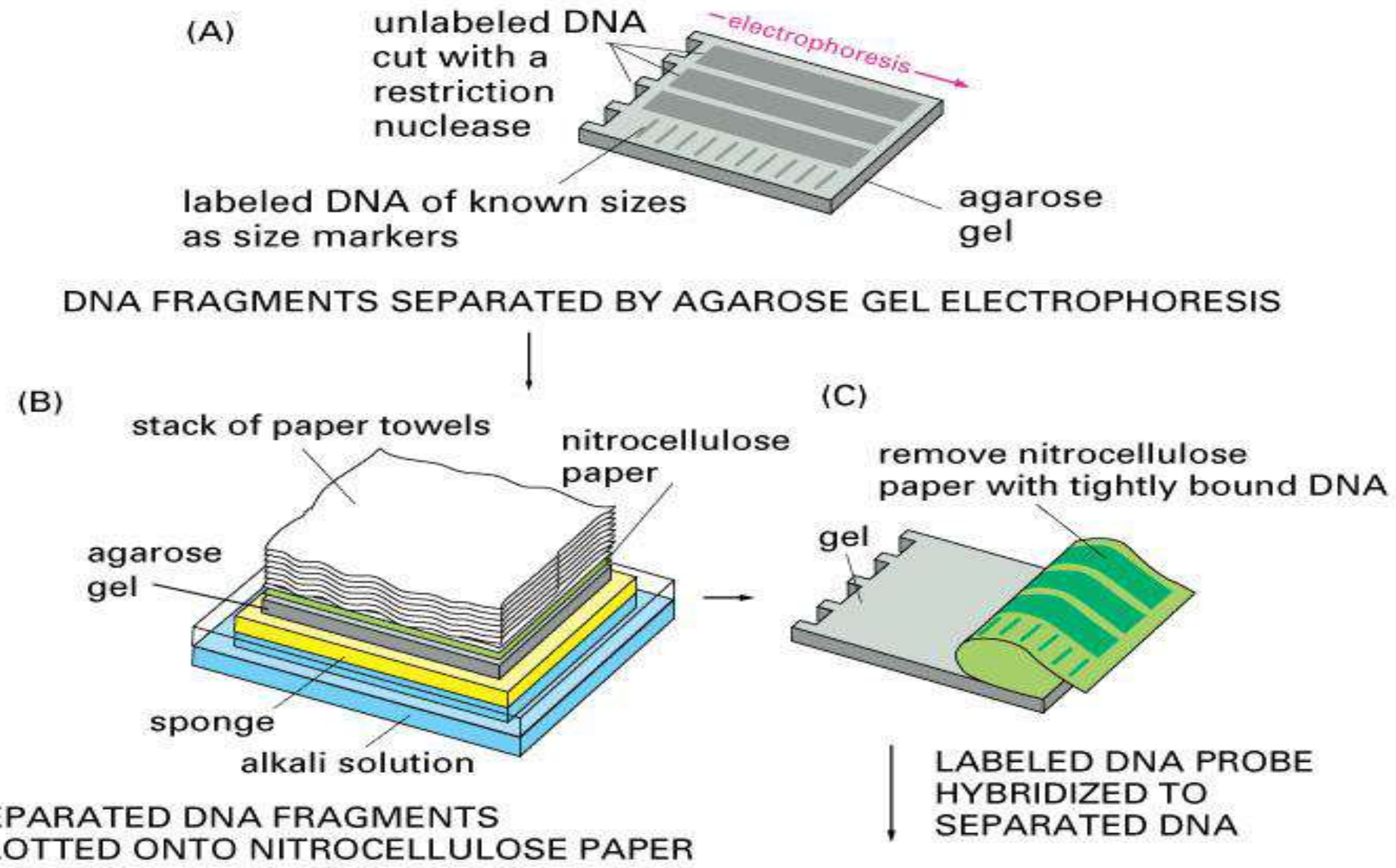
Chemiluminescence, chemifluorescence or chromogenic techniques are used for the detection of non-isotopic probes. Biotinylated probes are detected with streptavidin conjugated to a reporter enzyme.

Enzyme-linked immunoassay using an antibody conjugated alkaline phosphatase is used to detect digoxigenin labeled probes.

The detection of fluorescein labeled probes is carried out using antifluorescein antibodies conjugated to alkaline phosphatase or horseradish peroxidase.

Southern blotting and hybridization



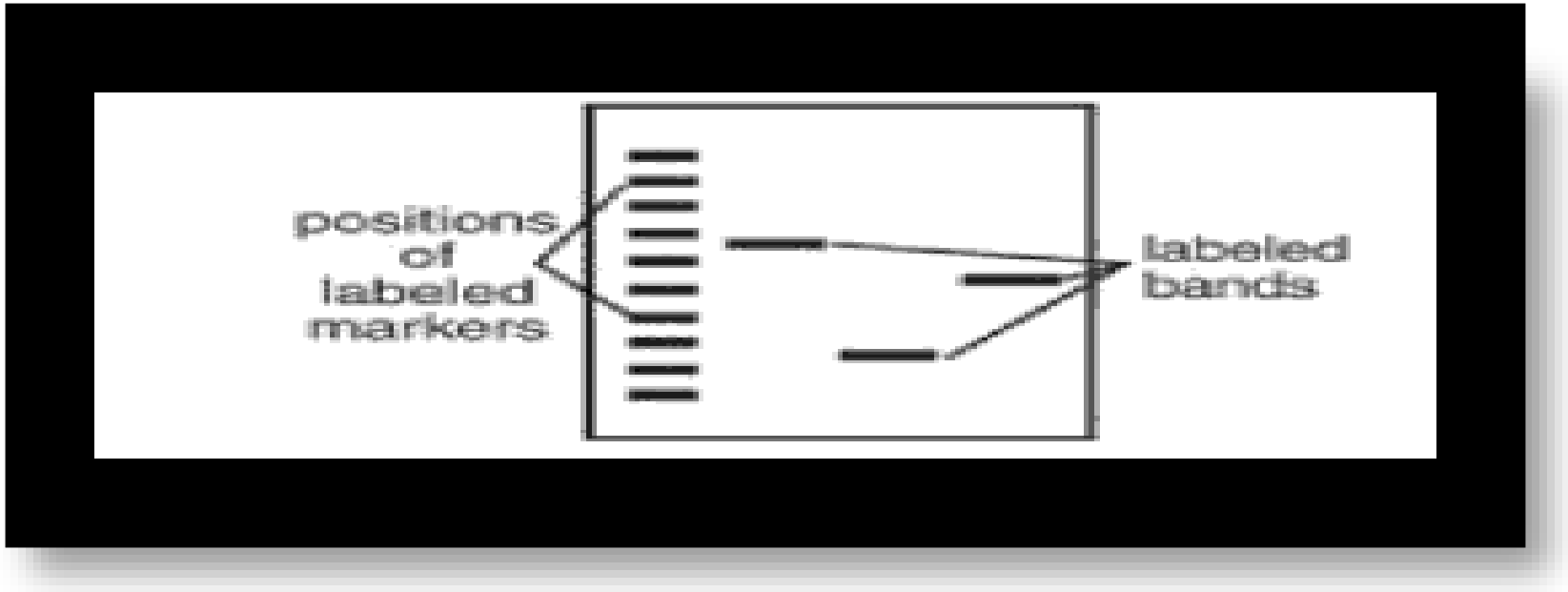

(A)
unlabeled DNA cut with a restriction nuclease
electrophoresis
labeled DNA of known sizes as size markers
agarose gel
DNA FRAGMENTS SEPARATED BY AGAROSE GEL ELECTROPHORESIS
(B)
stack of paper towels
nitrocellulose paper
agarose gel
sponge
alkali solution
SEPARATED DNA FRAGMENTS BLOTTED ONTO NITROCELLULOSE PAPER
(C)
remove nitrocellulose paper with tightly bound DNA
gel
LABELED DNA PROBE HYBRIDIZED TO SEPARATED DNA

Autoradiograph

is an image on an x-ray film

•The location of the probe is revealed by converting a colorless substrate to a colored product that can be seen orgives off light which will expose X-ray film.

•The bands indicate the number and size of the DNA fragments complementary to the probe.



Application of southern blotting

Main functions

Detect the specific DNA sequence (gene) of interest. Determine the length of the restriction fragment carrying the sequence.

Detect the restriction site.

Identify specific DNA sequences in DNA samples

- Isolate desired DNA for construction of rDNA.
- Identify mutations, deletions and gene rearrangements.
- In GMOs, used for testing to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism.
- Phylogenetic analysis
- To determine the number of copies of a particular DNA sequence
- In DNA Fingerprinting for:
  - Paternity & Maternity tests
  - Forensics
  - Personal/ Bio identification

Diagnosis of human disease

Detect point mutation, gene rearrangement or gene amplification

- Mutated gene change in the size (hemophilia A)
- Gene rearrangement change in size and pattern (leukemia)
- Amplification increase in gene copy number (Charcot-Marie- Tooth syndrome)

Disadvantages of southern blotting

More expensive than most other tests.

Complex and labor-intensive.

Time consuming an combersome.

Requires a large amount of targeted DNA.

Colony hybridization

Colony hybridization, also known as replica plating, allows the screening of colonies plated at high density using radioactive DNA probes. This method can be used to screen plasmid or cosmid based libraries.

1) **Preparation of Master plate:** First, inoculate the bacterial cell suspension on the solid agar medium to prepare the master plate. After the inoculation, the number of bacterial colonies will develop with different plasmids which refer as “Master or Reference plate”.

2) **Formation of replicas over a nitrocellulose filter:** Transfer the bacterial cells from the master plate on to the membrane or filter by the means of “Nitrocellulose filter”. Press the nitrocellulose filter paper over the surface of the master plate. This compression of the filter membrane will form replicas or copies of the bacterial cells as that of the master plate.

3) **Treatment of filter paper with SDS:** Treat the nitrocellulose filter paper with the detergent like SDS (Sodium dodecyl sulfate) to lyse the bacterial cells.

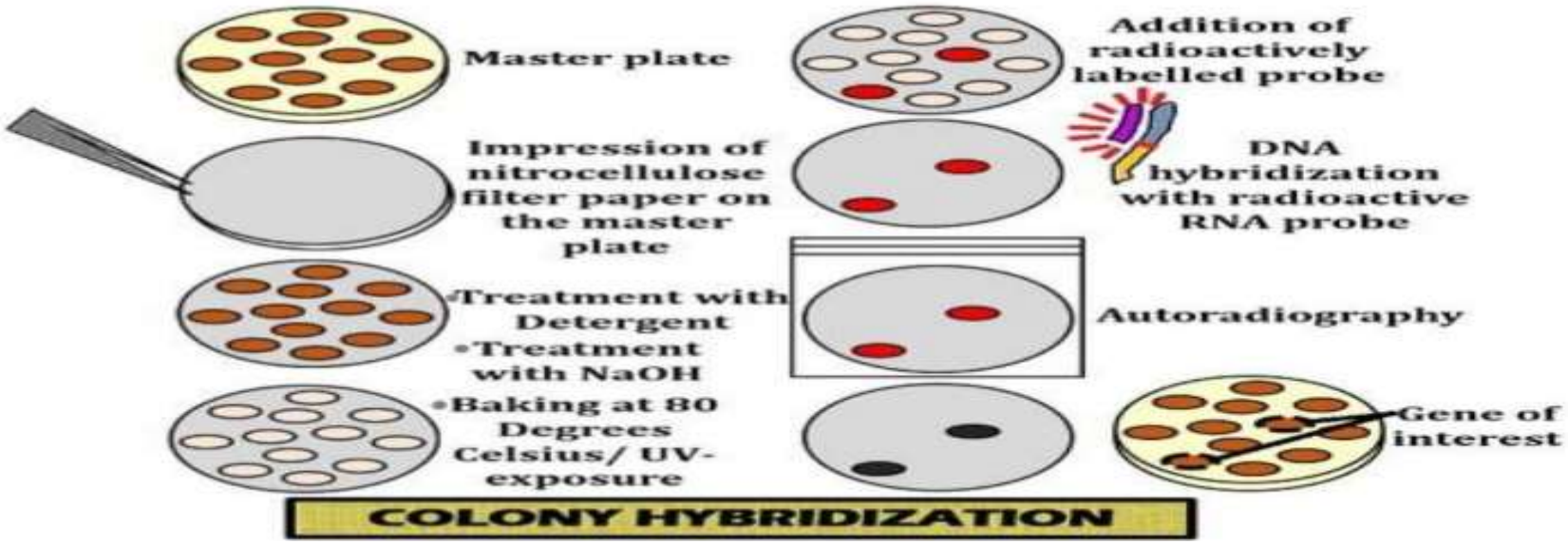
4) **Treatment of filter paper with alkali:** Treat the filter paper with the alkali like sodium hydroxide in order to separate the DNA into single strands.

5) **Fixation of DNA onto the filter paper**: To fix the DNA onto the nitrocellulose filter paper, either bake the filter paper at 80 degrees Celsius or expose it to the UV light.

6) **Addition of radioactive probe:** Hybridize the nitrocellulose filter paper containing imprints of the plasmid DNA by the addition of radioactive RNA probe. This radioactive RNA probe will code the desired gene of sequence from the bacterial cells.

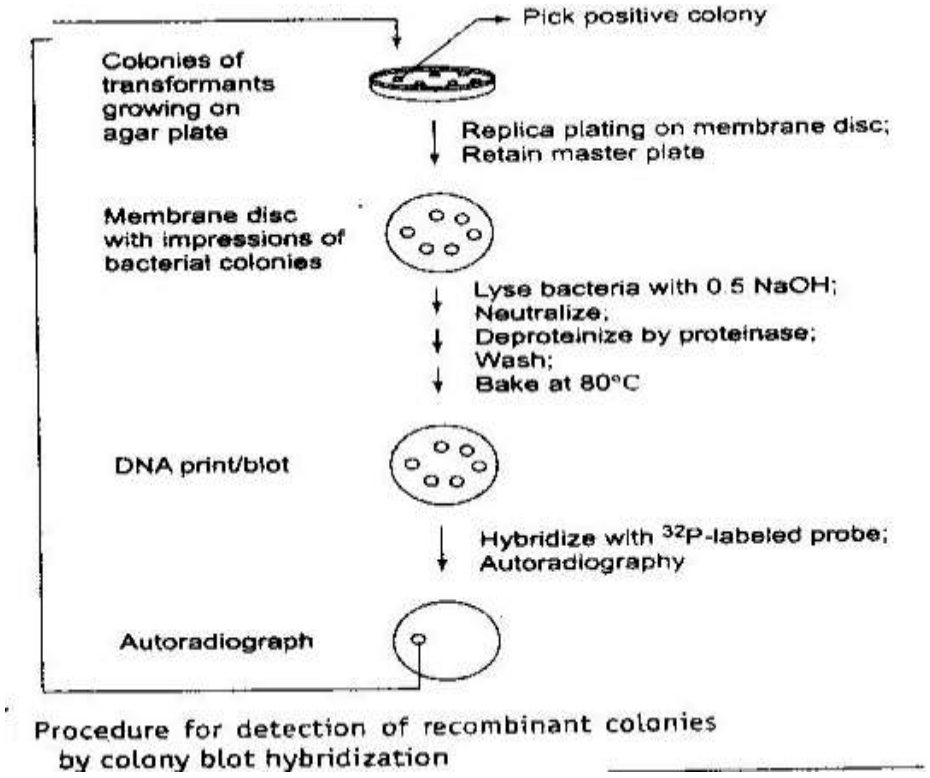
7) **Washing and Autoradiography:** Wash the filter paper to remove unbound probe particles. After that, expose the nitrocellulose filter paper to the X-ray film by the method refer as “Autoradiography”. The colony which will appear after autoradiography will refer as “Autoradiogram” which carry the genes of interest.

8) **Identification of the desired gene**: Compare the developed autoradiogram with the master plate to identify the colonies containing a gene of interest. The cells which contain the desired gene can grow in the liquid medium and can further process for the isolation of recombinant plasmid DNA

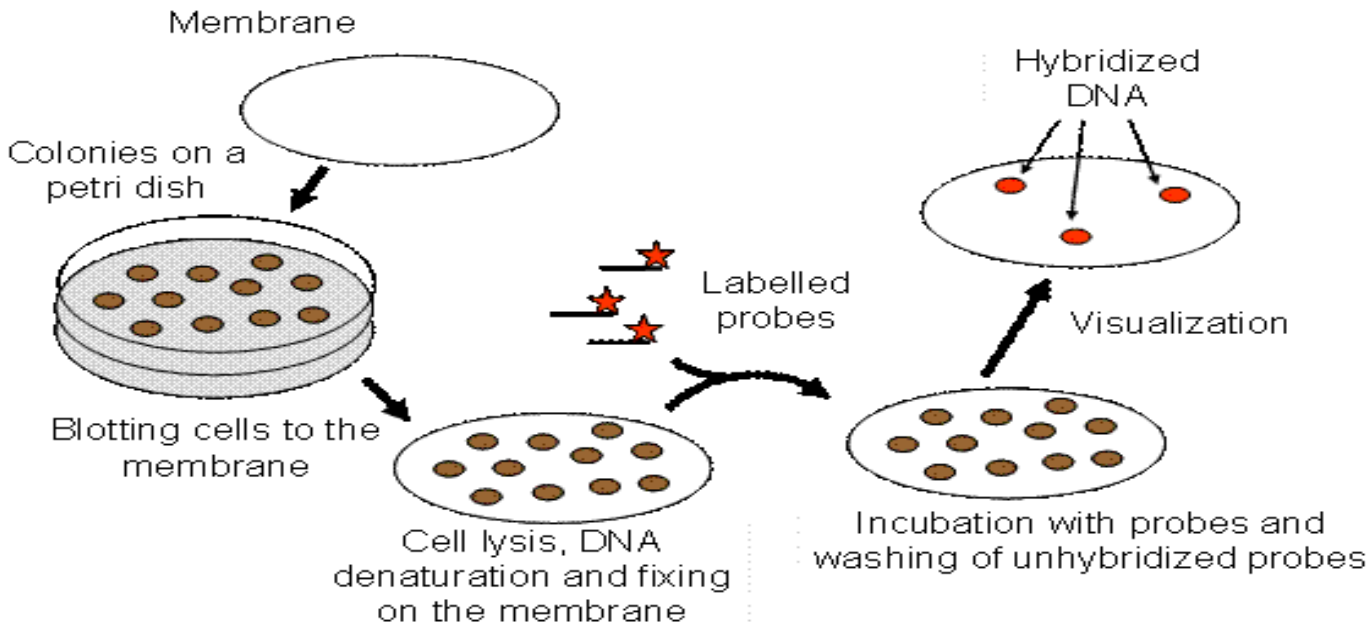


Colony blotting and hybridization

- Applied to nucleic acid released from blotted microbial colonies
- The most commonly used and rapid method of library screening as it can be applied to very large number of clones



Procedure for detection of recombinant colonies by colony blot hybridization



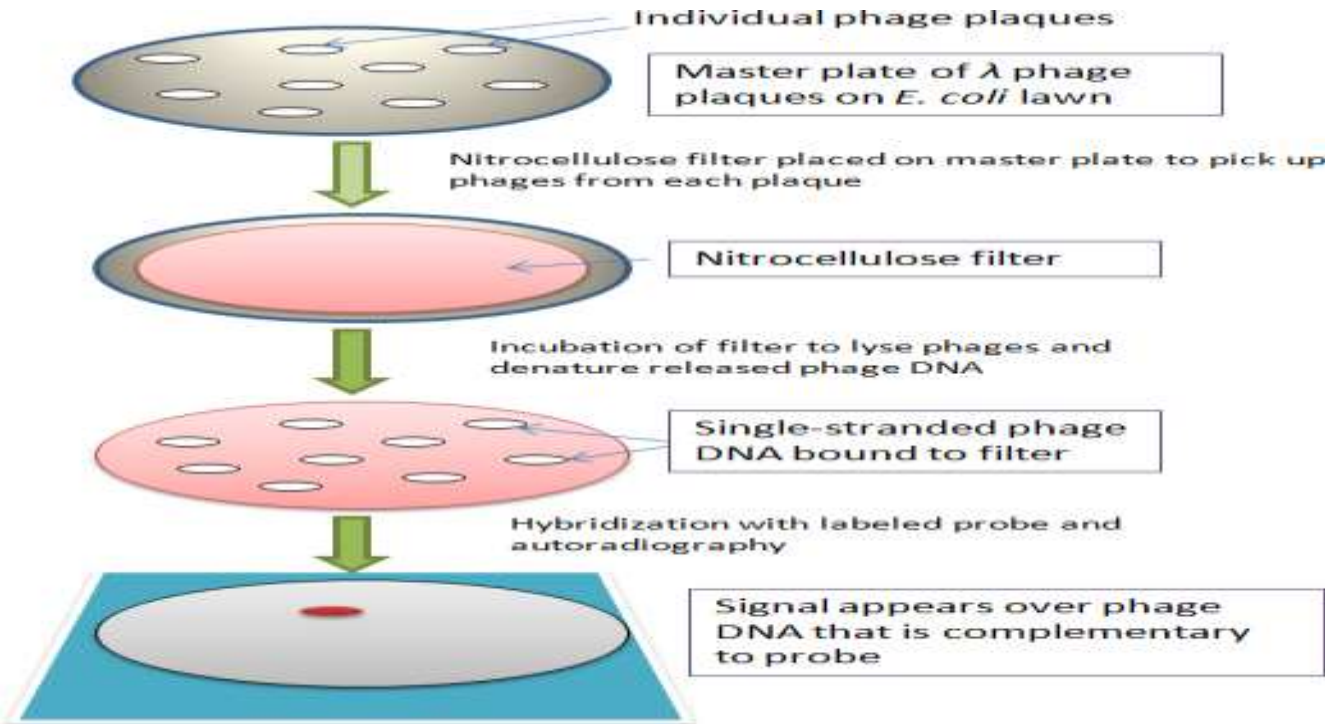
Plaque hybridization

Plaque hybridization, also known as *Plaque lift*, was developed by Benton and Davis in 1977 and employs a filter lift method applied to phage plaques. This procedure is successfully applied to the isolation of recombinant phage by nucleic acid hybridization and probably is the most widely applied method of library screening. The method of screening library by plaque hybridization is described below-

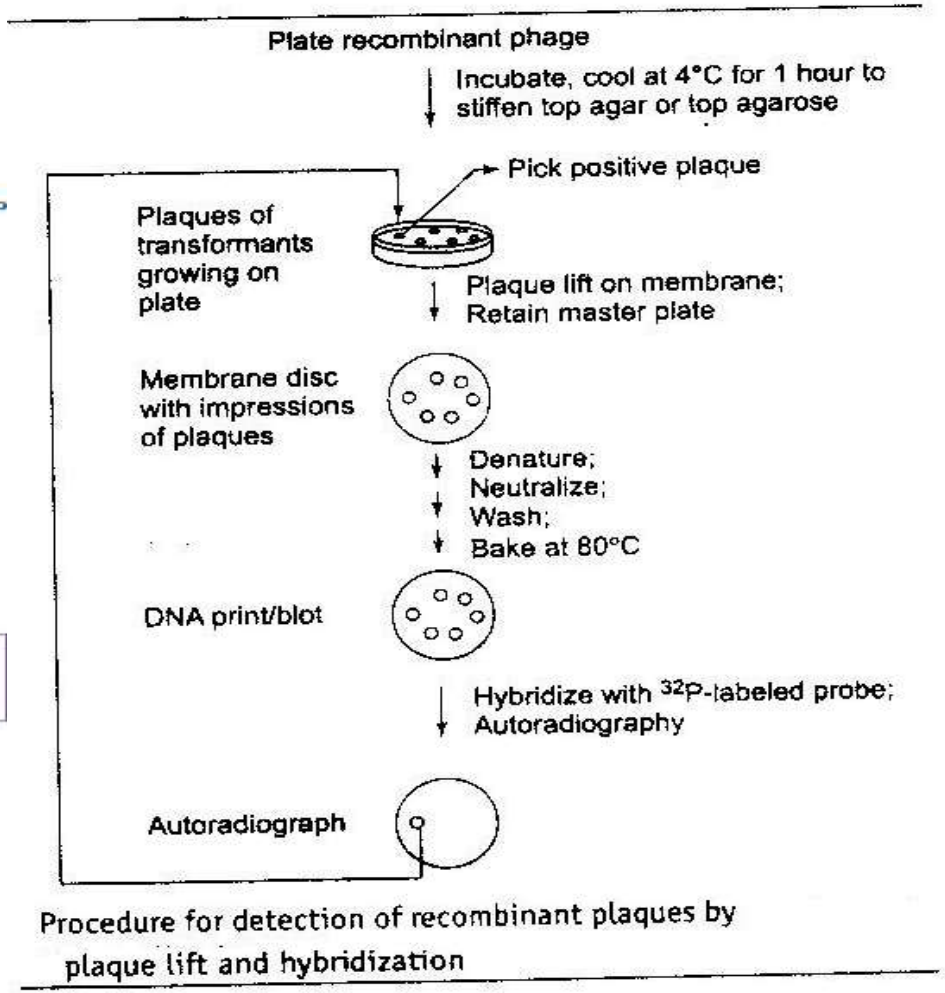
- The nitrocellulose filter is applied to the upper surface of agar plates, making a direct contact between plaques and filter.
- The plaques contain phage particles, as well as a considerable amount of unpackaged recombinant DNA which bind to the filter.
- The DNA is denatured, fixed to the filter, hybridized with radioactive probes and assayed by autoradiography.

Advantages

- This method results in a 'cleaner' background and distinct signal (less background probe hybridization) for λ plaque screening due to less DNA transfer from the bacterial host to the nitrocellulose membrane while lifting plaques rather than bacterial colonies.
- Multiple screens can be performed from the same plate as plaques can be lifted several times.
- Screening can be performed at very high density by screening small plaques. High-density screening has the advantage that a large number of recombinantclones can be screened for the presence of sequences homologous to the probe in a single experiment.



4-5.2.1(b). Schematic process for screening libraries by Plaque hybridization.



Plaque lift and hybridization

NORTHERN BLOTTING

The **northern blot is a technique used in molecular** biology to study gene expression by detection of RNA (or isolated mRNA) in a sample.
Developed by Alwnie and his colleagues in 1979.
This method was named for its similarity to the technique known as a Southern blot.
Steps of northern blotting
No need to digest RNA with restriction enzymes.
Extraction of RNA:
The RNA sample can be:
i. total RNA isolated from particular samples
ii. RNA containing poly(A) tails, i.e: messenger RNA(mRNA)
Extraction of total RNA from a homogenized tissue sample or from cells.
• mRNA isolated through the use of oligo (dT) cellulose chromatography to isolate only those RNAs with a poly(A) tail.

Gel electrophoresis

RNA samples are then separated by gel electrophoresis.
In gel electrophoresis the mixture of mRNA molecules separated/denatured to small fragments/molecules according to their size using an electric field.

Formaldehyde :

Formaldehyde is used to unfragment the branched RNA molecule to simple linear one and to prevent it form coiling again.

Blotting

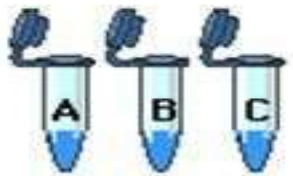
The transfer or blotting is the step in which the mRNA from the electrophoresis gel will be transferred onto a nylon membrane.
Traditionally, a nitrocellulose membrane is used, although nylon or a positively charged nylon membrane may be used.
Nitrocellulose typically has a binding capacity of about 100μg/cm, while nylon has a binding capacity of about 500 μg/cm. Many scientists feel nylon is better since it binds more and is less fragile.
RNA,s will be covalently link to membrane.

Gel Electrophoresis

- **Conclusions**

1. Make gel.

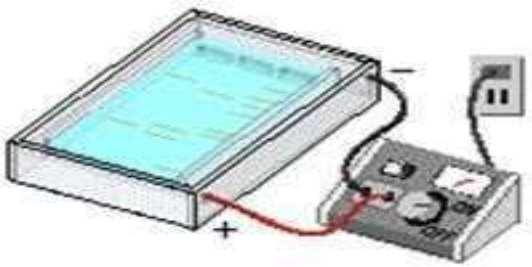
2. Obtain prepared RNA samples.



3. Load samples into gel.

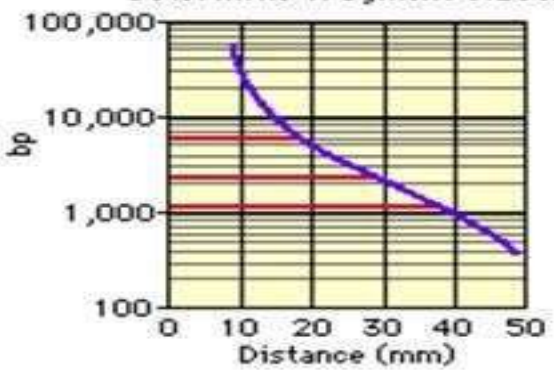
RNA = negatively charged

4. Separate fragments by electrophoresis.

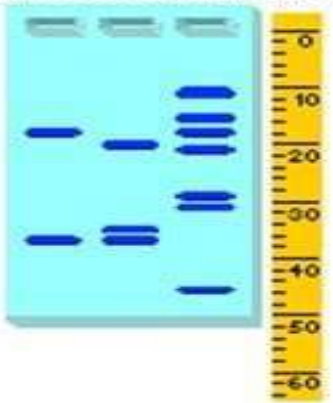


correlate distance to size

6. Prepare a standard curve. Determine fragment sizes.

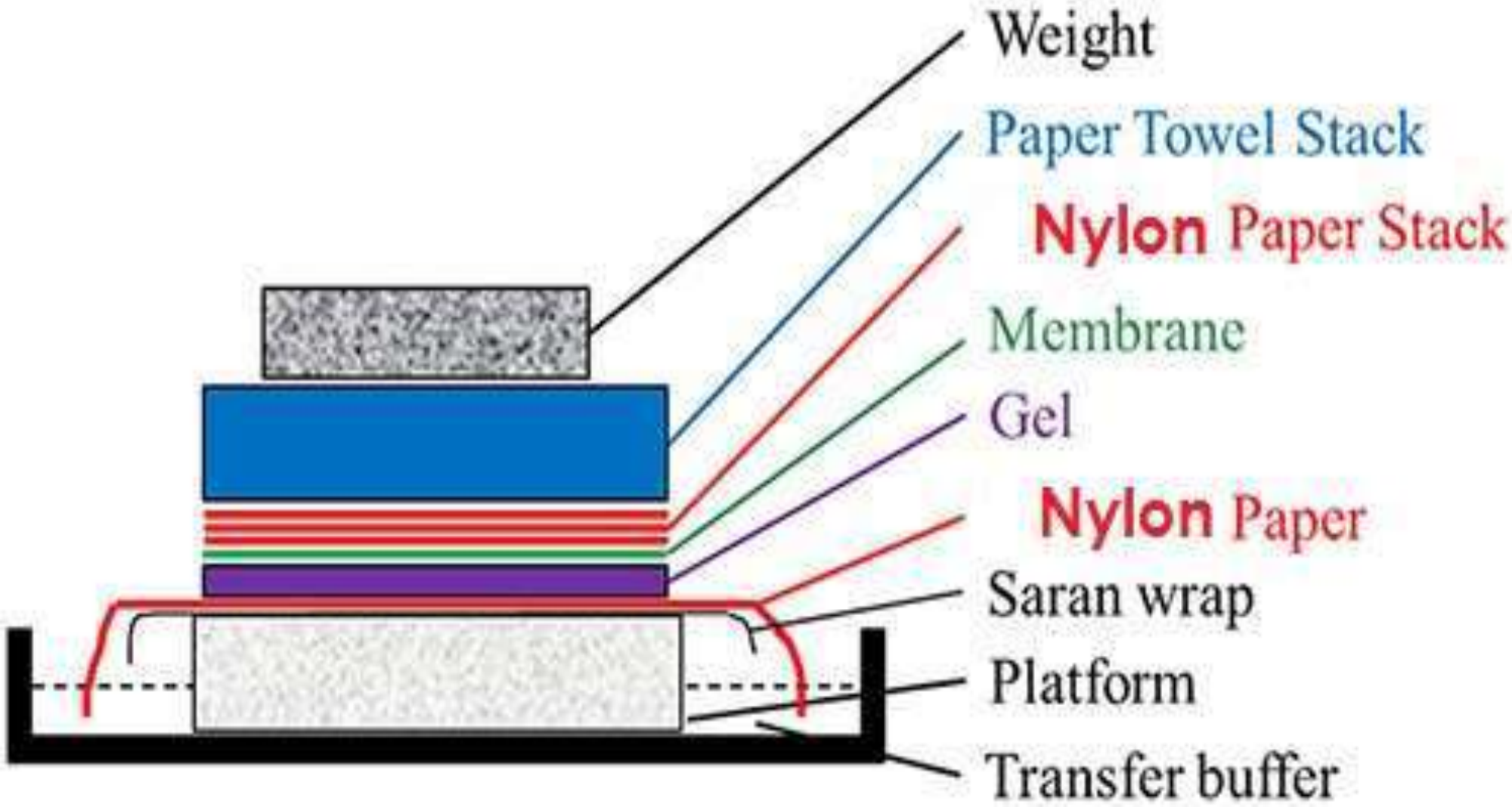


5. Stain RNA fragments and measure distances.



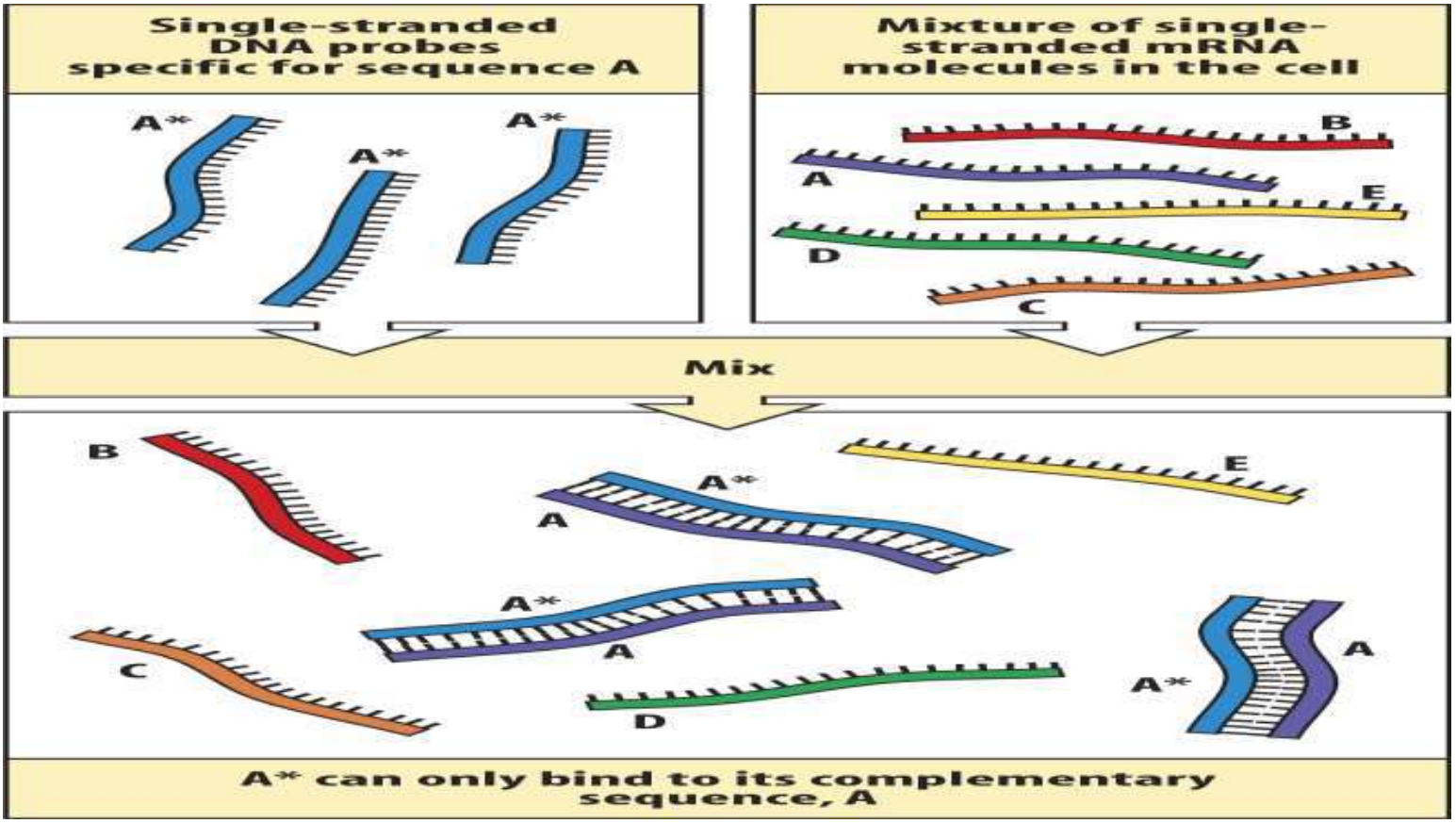
smaller fragments travel faster

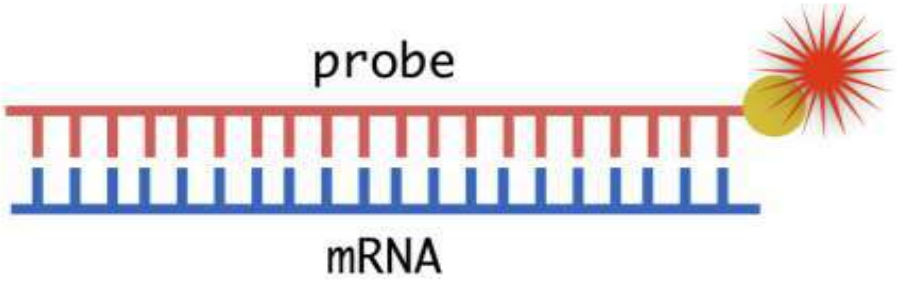
Blotting setup for northern



Hybridization

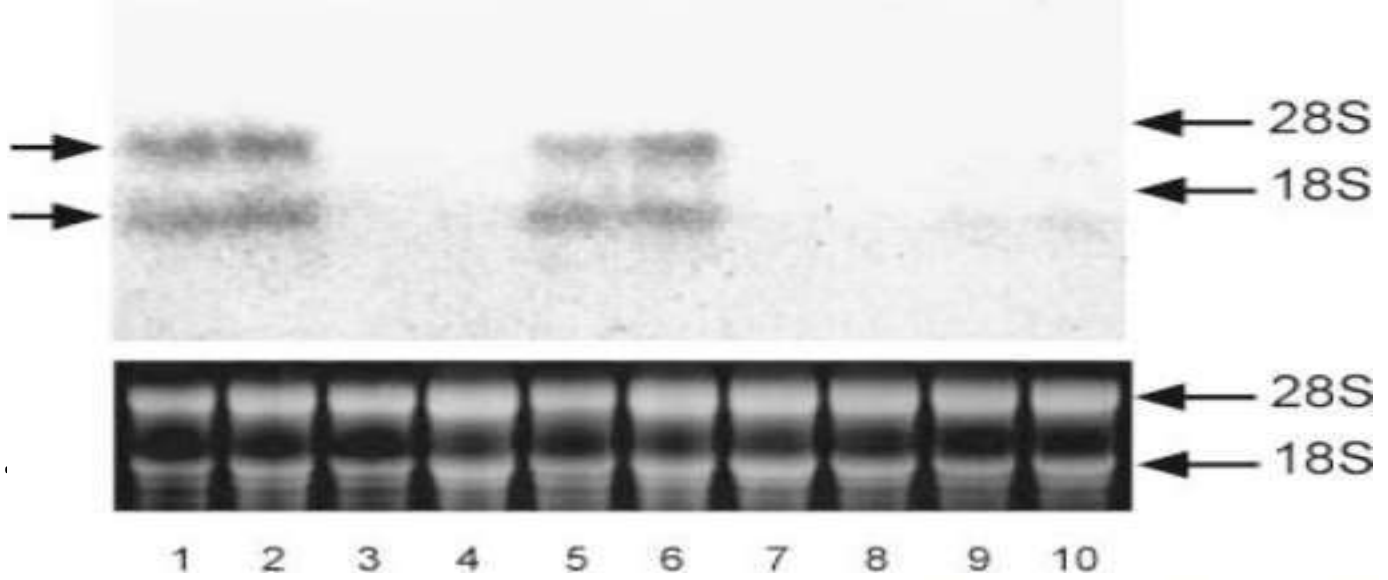
In molecular biology hybridization means the process of forming a double stranded nucleic acid from joining two complementary strands of DNA (or RNA).
Incubate membrane with labeled DNA or RNA probe with target sequence



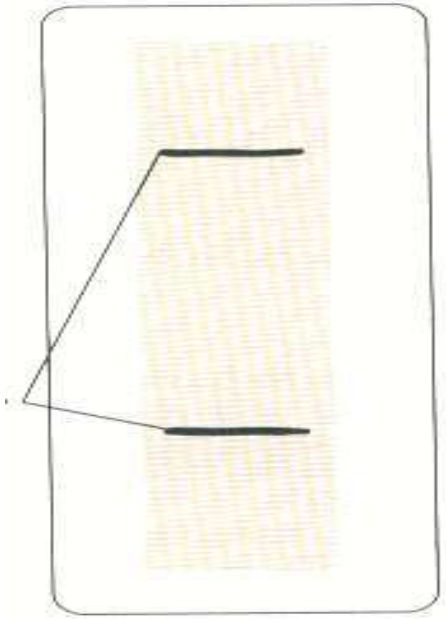


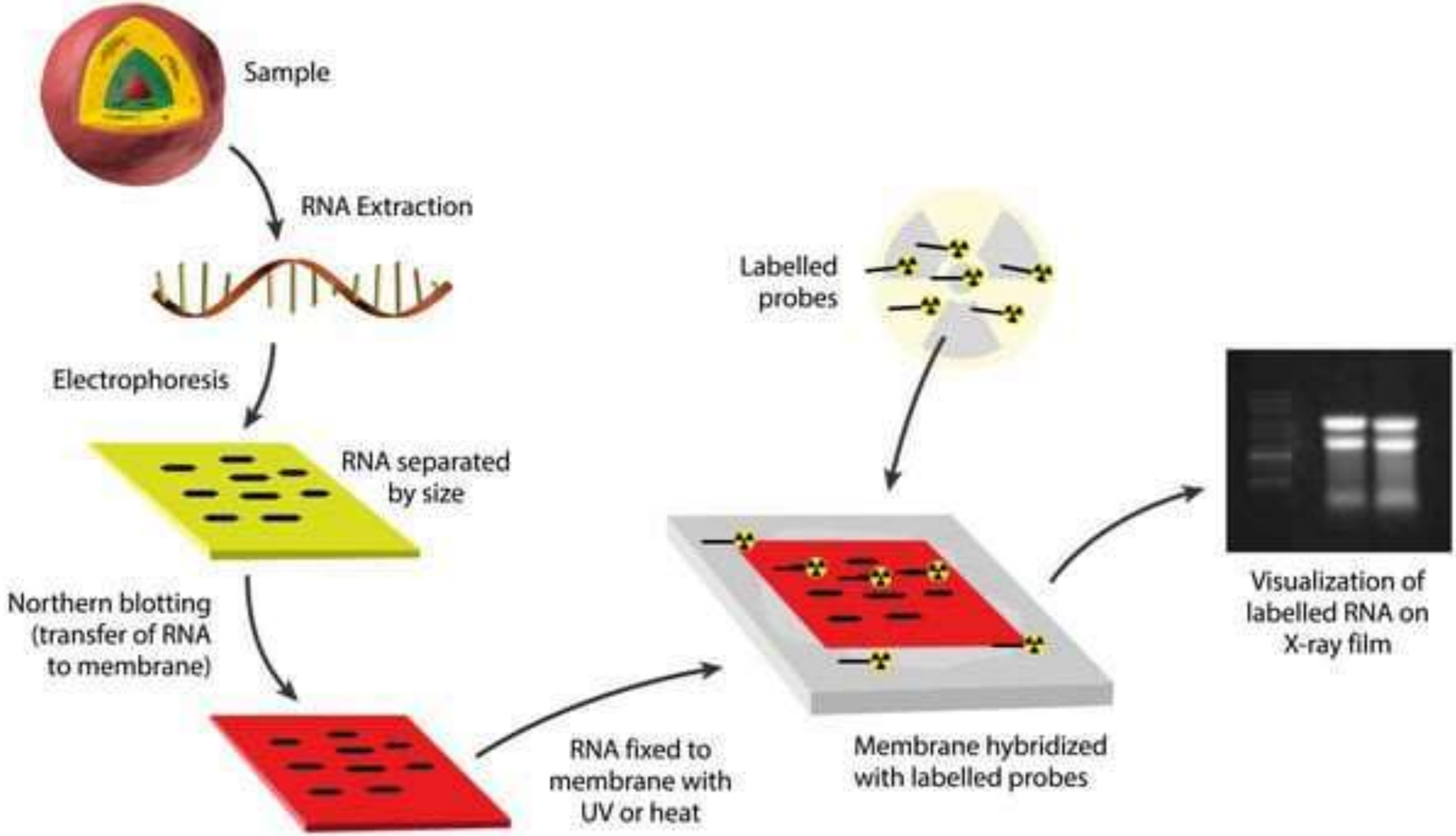
Washing And Visualization

Wash Nylon from excess of probe & dry.
Place Nylon sheet over x-ray film.
X-ray film darkens where the fragments are
complementary to the radioactive probes.
Advantages/Application
Observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment.
• Used to show overexpression of oncogenes and down regulation of tumorsuppressor genes in cancerous cells.
• Detecting a specific mRNA in sample, used for screening recombinants which are successfully transformed with transgene.
• mRNA splicing studies

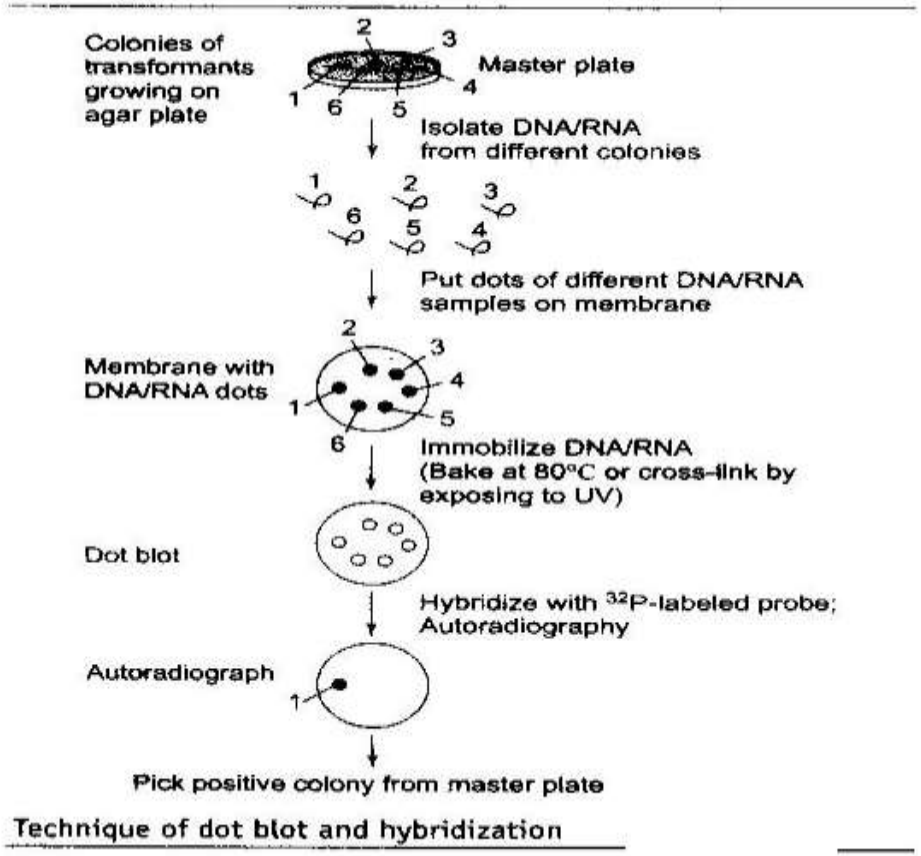


Main functions
Detect mRNA transcriptional activity
Quantifying the transcription
Determine the size of the mRNA
Determine mRNA level

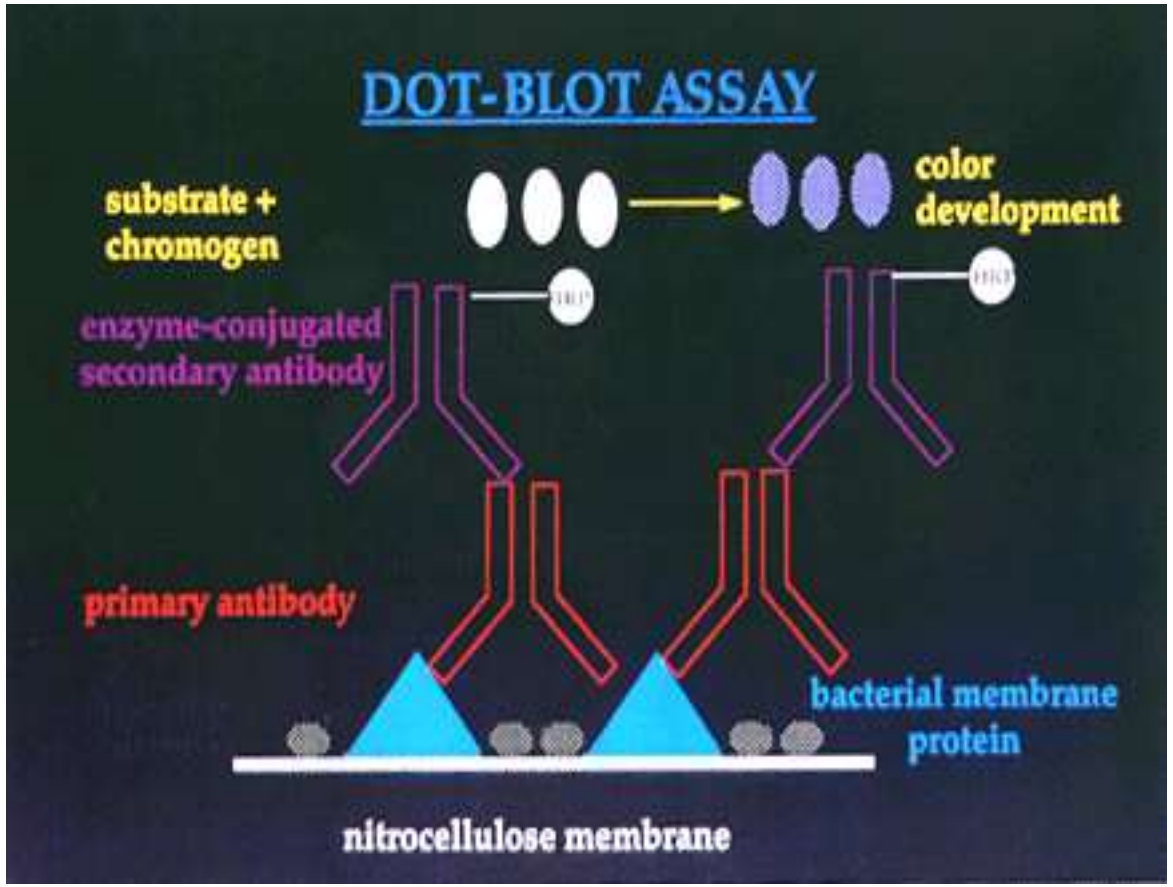



Sample
RNA Extraction
Electrophoresis
RNA separated by size
Northern blotting (transfer of RNA to membrane)
RNA fixed to membrane with UV or heat
Labelled probes
Membrane hybridized with labelled probes
Visualization of labelled RNA on X-ray film

DNA and RNA dot blot



Technique of dot blot and hybridization



Protein Structure/Function Based Techniques

When a DNA library is constructed using expression vectors, it permits a range of alternative techniques to be employed, each of which exploits some structural or functional property of the gene product as each individual clone can be expressed to yield a polypeptide

These approaches are very important in cases where the DNA sequences of target clone is completely unknown and enough information is not available to design a suitable probe or set of primers

Screening by determination of protein activity

If the target gene produces an enzyme that is not normally made by the host cell, an *in situ* (direct plate) assay can be developed to identify members of a library that carry the particular gene encoding that enzyme

For example, the gene for α-amylase, β-glucosidase and many other enzymes from various organisms have been isolated in this way

Screening by gain of function

In many cases, the function of the target gene is too specific, which does not work in bacterial or yeast expression host and even in a higher eukaryotic system

Therefore an alternative method has been devised in which positive clones can be identified on the basis of gain of function conferred on the host cell

This gain of function may be a selectable phenotype that permits cells having the corresponding clone to be positively selected

Gain of function strategy of screening can also be employed for analysis of transgenes

Phage display

In phage display system, a library of proteins or peptides is expressed as fusion proteins with a surface-displayed coat protein of a filamentous phage such as M13

This technique is called phage display because it involves the display of proteins on the surface of M13 bacteriophage

Proteins expressed in this manner can be probed using strategies similar to those utilized for screening conventional bacterial expression libraries

Phage display is mainly used:

To identify and analyze features on the surface of proteins

To isolate new ligands that bind to particular amino sequences

To improve the affinity and specificity of the interaction between ligands and their target structures

Protein blotting techniques

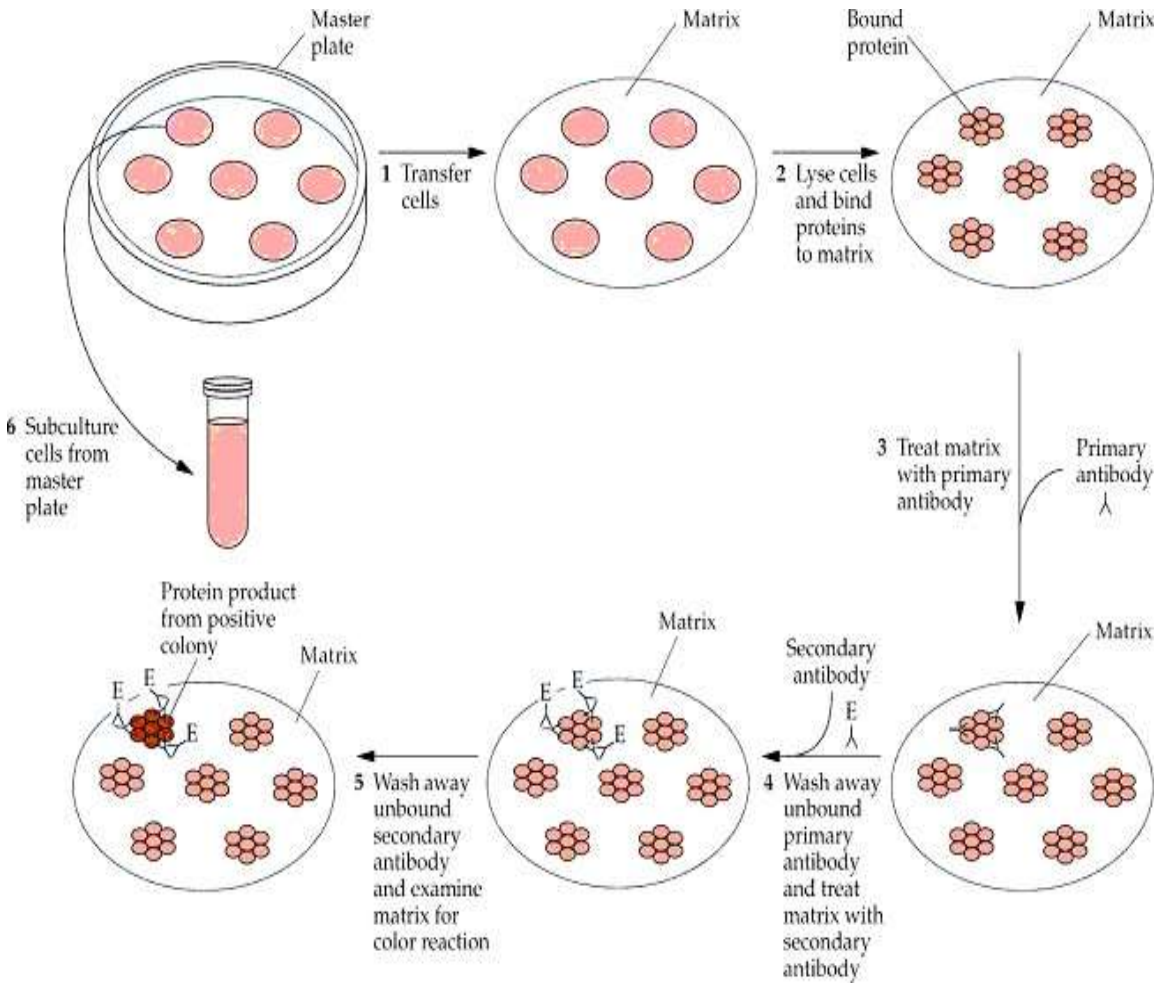
Similar to nucleic acid blotting, protein blotting techniques are also used for detection of proteins

These include :-

- **Immunological screening**
- **Plus-minus screening**
- **Western blotting**
- **Southwestern blotting**
- **Northwestern blotting**

Screening by Immunological assay

The desired gene can also be identified by the activities of the encoded gene product.



1. The cells are grown as colonies on master plates which are transferred to a solid matrix (i.e., nitrocellulose).

2. The colonies are then subjected to lysis and the released proteins bound to the matrix. These proteins are then treated with a primary antibody which specifically binds to the protein (acts as an antigen), encoded by the target DNA.

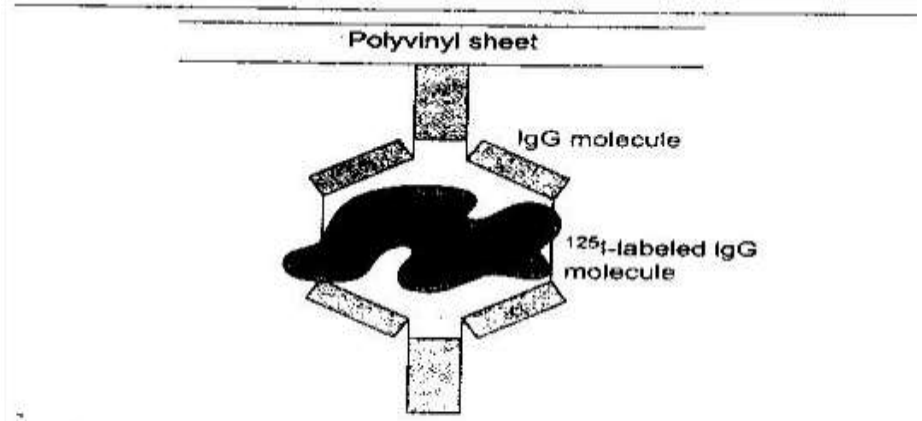
3. After removing the unbound antibody by washings, a second antibody is added which specifically binds to the first antibody.

4. Again the unbound antibodies are removed by washings. The second antibody carries an enzyme label (e.g., horse reddish peroxidase or alkaline phosphatase) bound to it. The detection process is so devised that as a colourless substrate it is acted upon by this enzyme, a coloured product is formed.

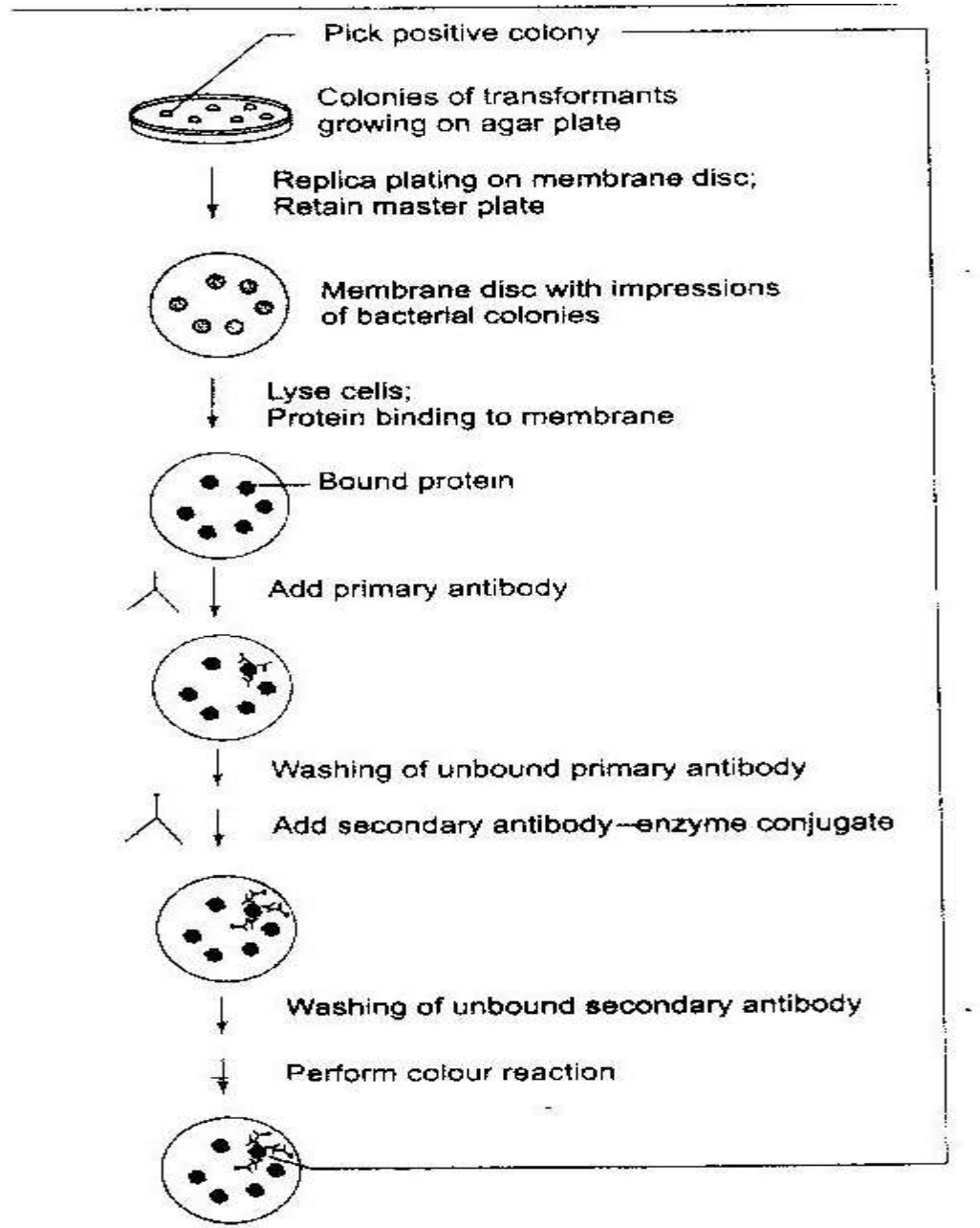
5. The colonies which give positive result (i.e., coloured spots) are identified. The cells of a specific colony can be sub-cultured from the master plate.

Immunological screening

It involves the use of antibodies that specifically recognize antigenic determinants on the polypeptide synthesized by a target clone
This is one of the most versatile expression cloning strategies because it can be used for any protein provided an antibody is available



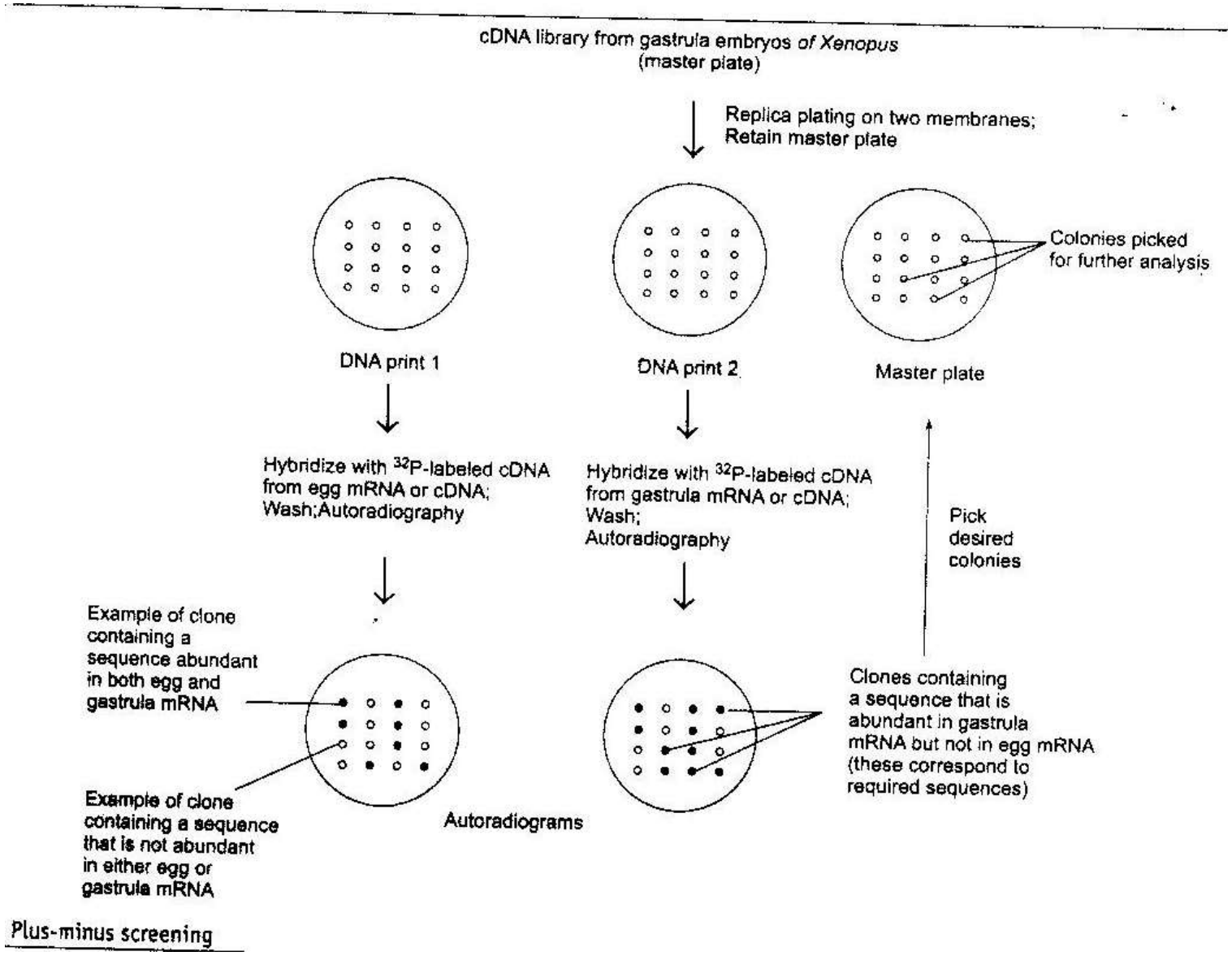
Antigen–antibody complex formation in the immunochemical detection



Immunological screening of a gene library

Plus-minus screening

Also known as differential screening, involves construction of cDNA library from cells expressing the gene of interest
Duplicate copies of the library are then screened separately with labeled cDNA probes synthesized from two preparations of mRNA
First, mRNAs are isolated from a cell type or tissue that expresses the gene(s) of interest in high abundance and the second from a cell type or tissue with low abundance of the target gene



Plus-minus screening

Southwestern and Northwestern blotting

South western blotting **: This technique combine the principles of southern and western blots and has been efficiently used for screening and isolation of clones expressing sequence specific DNA-binding proteins by using ss DNA probe**

North western blotting **: This technique is devised to isolate sequence specific RNA-binding proteins by using ss RNA probe**

Both of these techniques are most efficient when the oligonucleotides contain the binding sequence in multimeric form

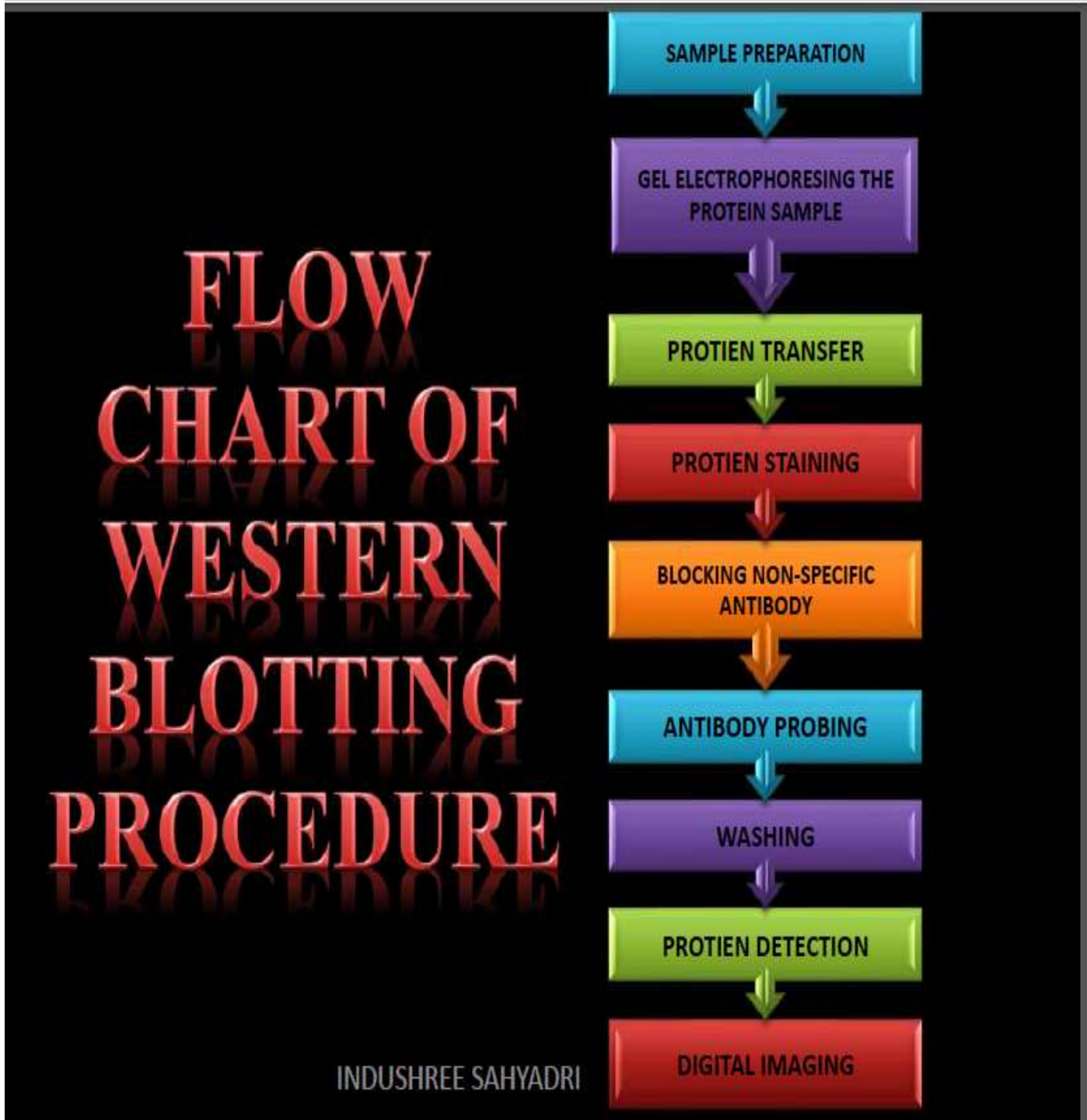
WESTERN BLOTTING

Western blotting is a widely used analytical technique in molecular biology to detect specific protein in a sample of tissue homogenate or extract. It works on the principle of gel electrophoresis.

Proteins are separated based on their size on polyacrylamide gel

The method originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute, Switzerland in 1979.

• The name western blot was given to the technique by W. Neal Burnette and is a play on the name Southern blot.



FLOW CHART OF WESTERN BLOTTING PROCEDURE

INDUSHREE SAHYADRI

Gel Electrophoresis

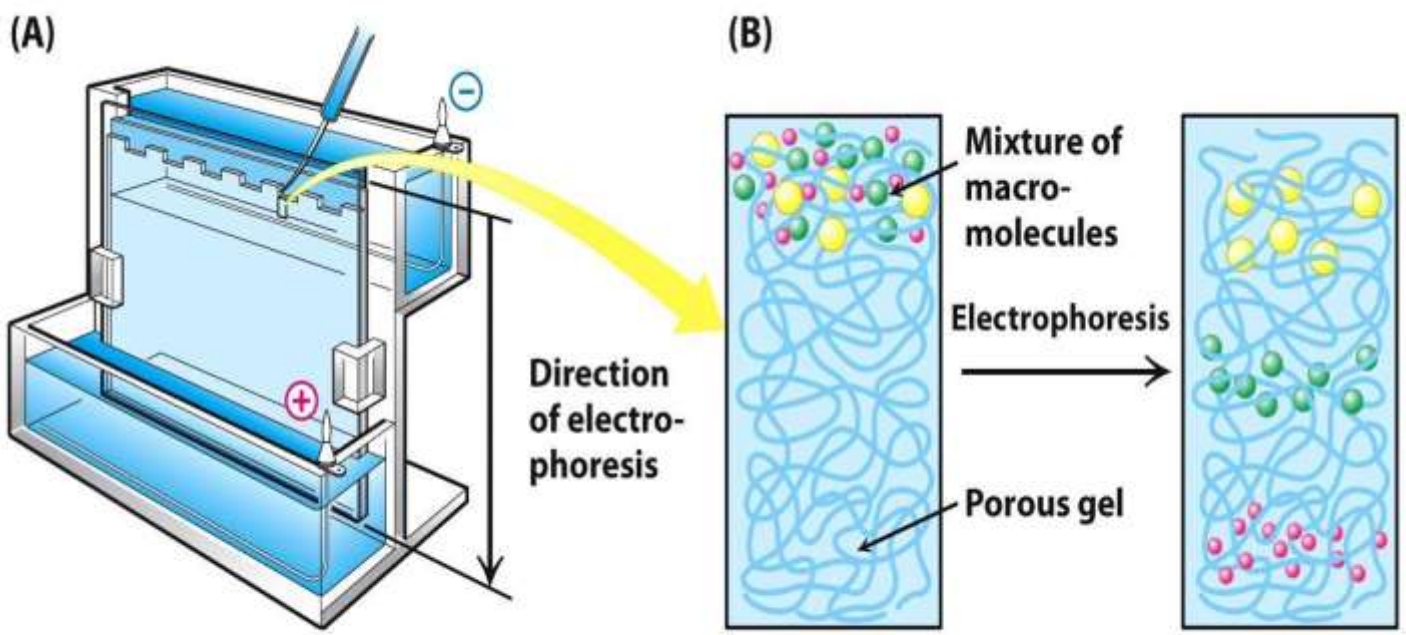
Electrophoresis is commonly used method for separating proteins on the basis of size, shape or charge.

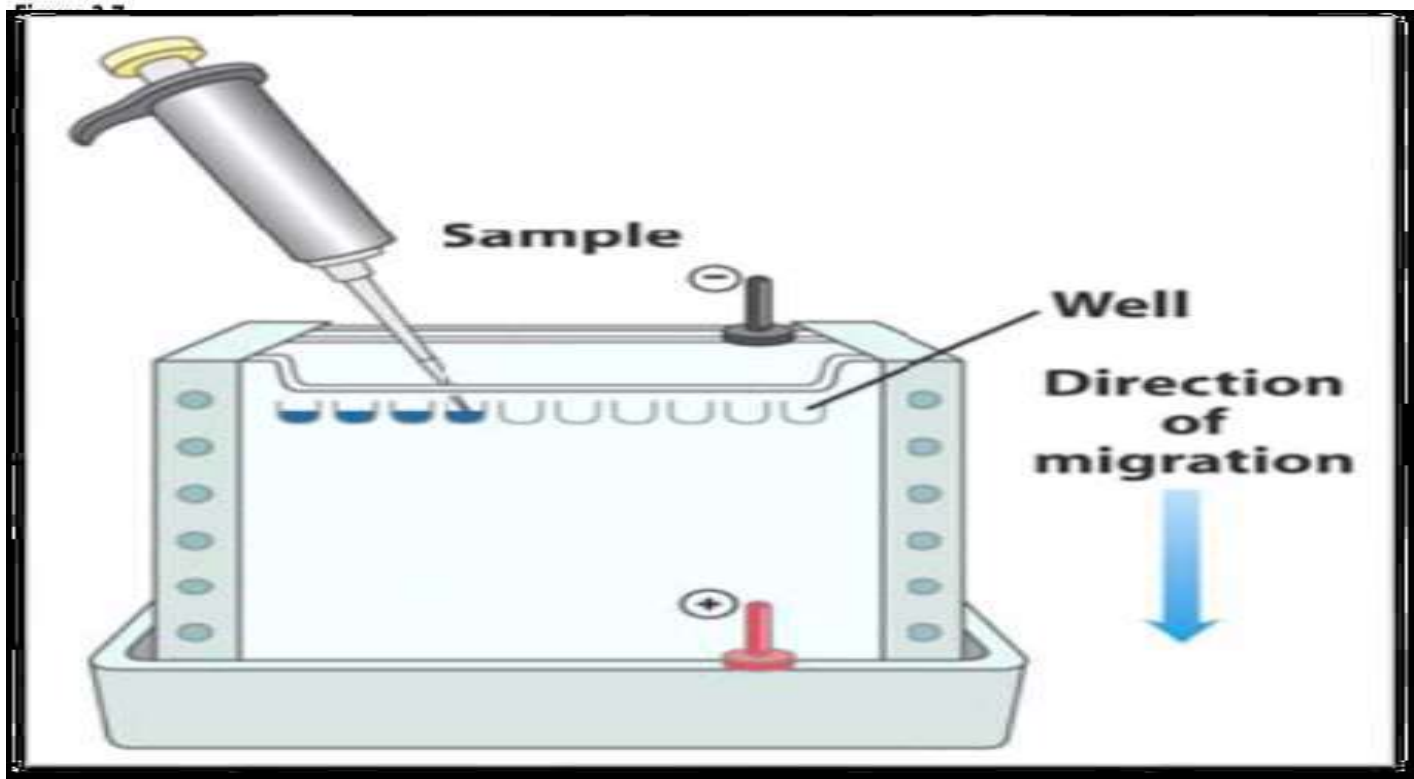
In Gel electrophoresis, protein of sample extract are separated according to their molecular weight.

I-Samples are loaded into separate wells

II-Run at 200 volts for 30-40 minutes

III-Running Buffer (pH 8.3)



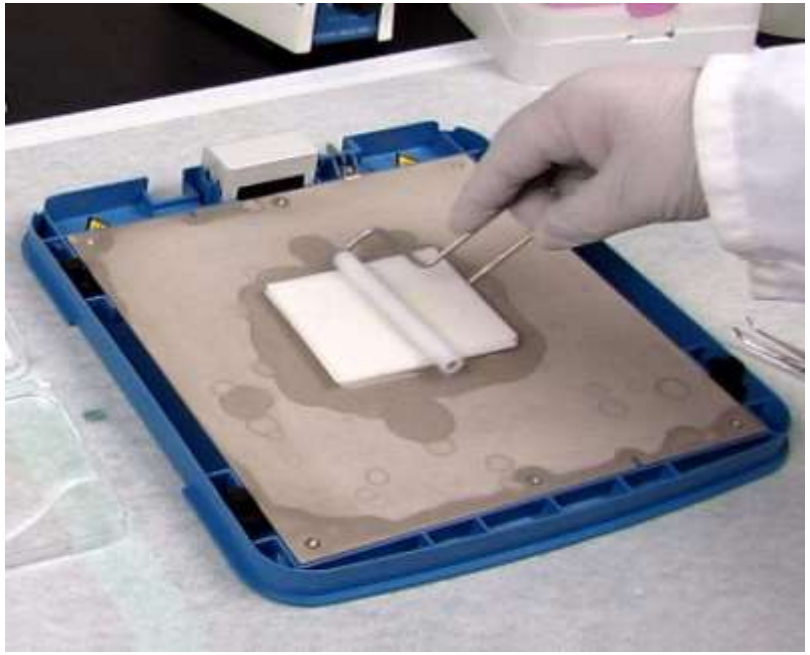


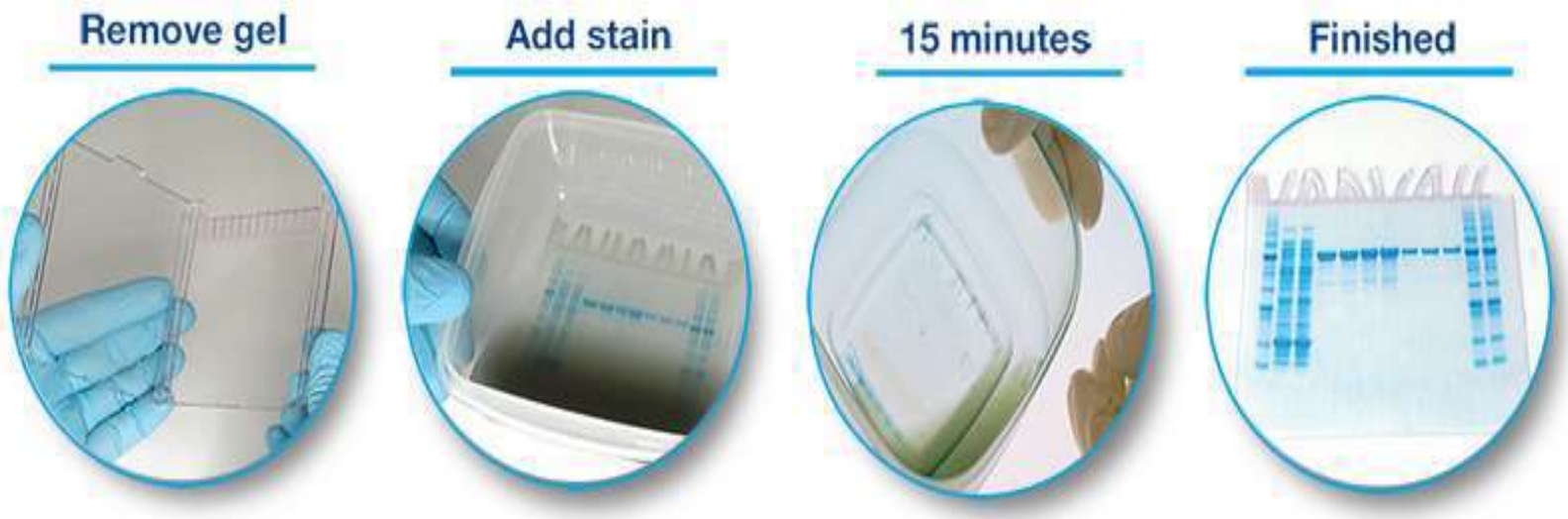
Protein transfer

On completion of the separation of proteins by polyacrylamide gel electrophoresis, the next step is to transfer the proteins from the gels to solid support membrane.

Protein staining

After gel electrophoresis, it may be necessary to confirm that all the proteins in the gel have been completely eluted. Proteins are usually stained with dyes such as coomassie blue, silver stain, or deep purple.





Blocking

For meaningful results, the antibodies must bind only to the protein of interest and not to the membrane.

Non-specific binding (NSB) of antibodies can be reduced by blocking the unoccupied sites of membrane with an inert protein or non-ionic detergent.

Blocking agents should possess greater affinity towards membrane than the antibodies.

Blocking agents

The most common blocking agents are:

a) Bovine serum albumin(BSA)
b) Non-fat milk
c) Casein
d) Gelatin
e) Dilute solution of Tween 20

Labeling with Primary Antibody

forms an antibody-protein complex with the protein of interest with specific Antibody.

Labeling with Secondary Antibody:

Is conjugated to HRP (horseradish peroxidase)
Acts as antibody against primary antibody
Antigens can be visualized through colored reaction

Washing

Unbound antibodies can cause high background and poor detection.

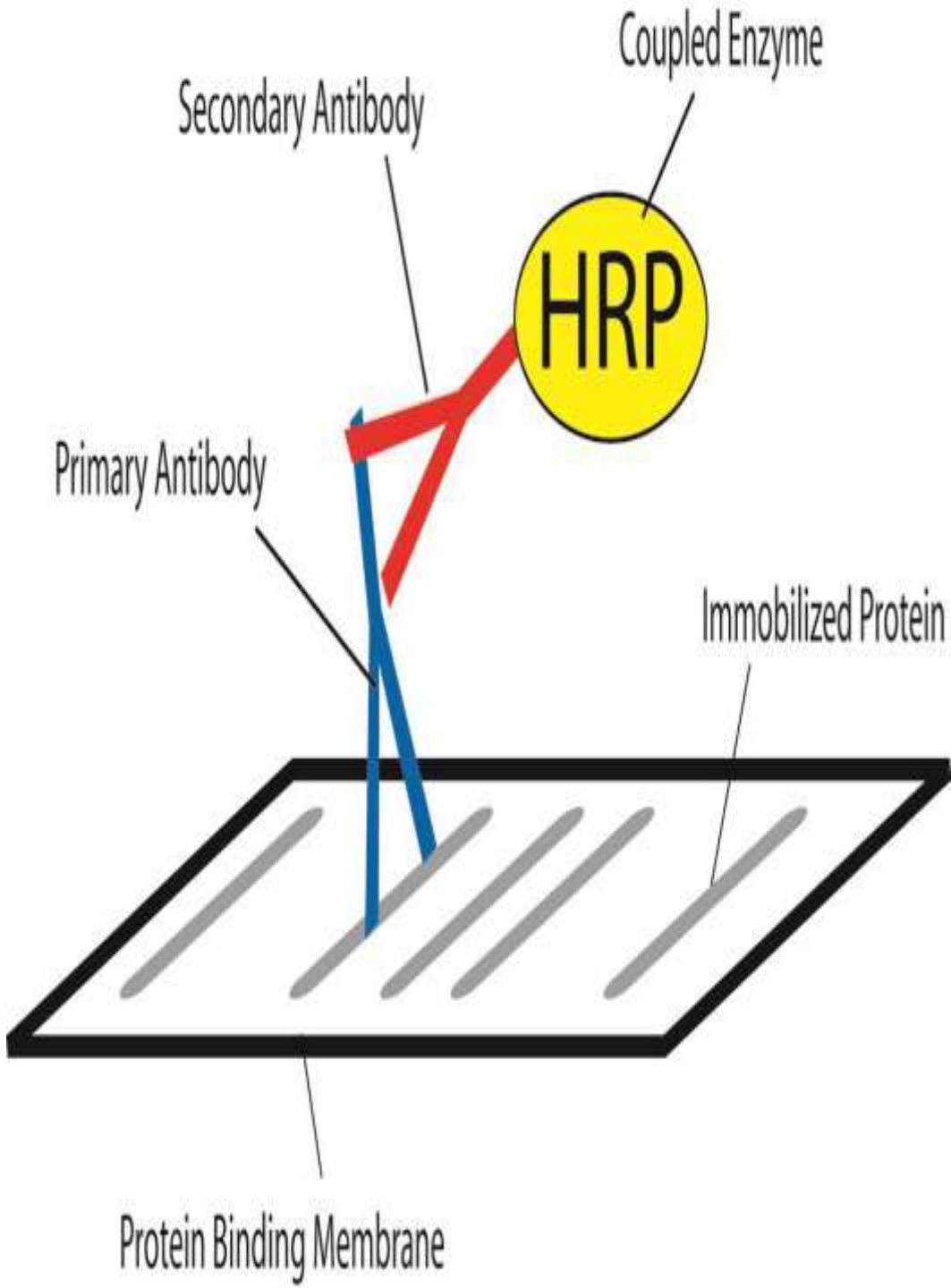
Hence Washing the blot removes unbound antibodies from the membrane.

A dilute solution of tween-20 in TBS or PBS buffer is commonly used for washing

Protein detection

After the unbound probes are washed away, the western blotting is now ready for detection of the probes that are labeled and bound to the protein of interest.

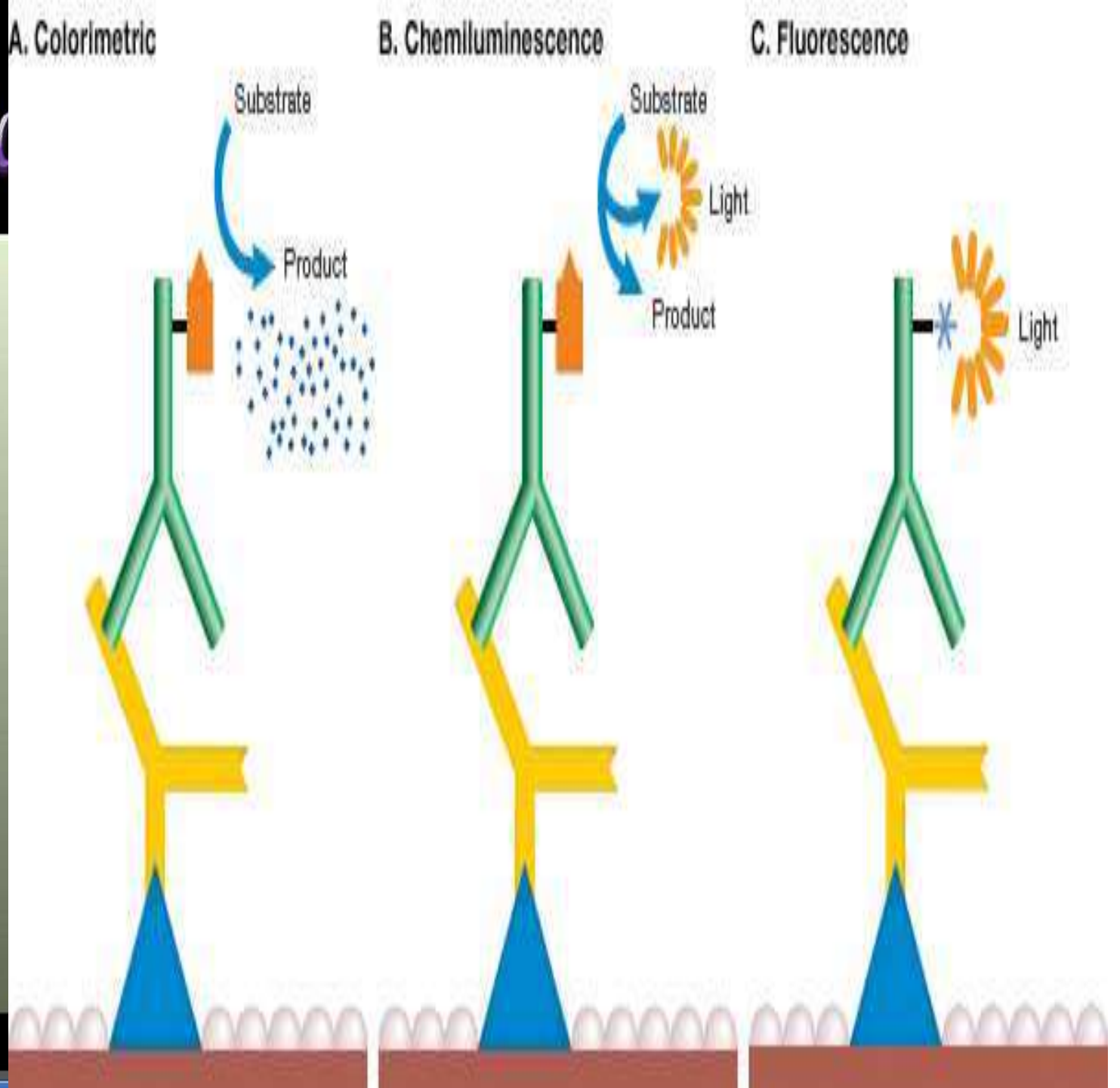
• Enzymes such as alkaline phosphatase(AP), & Horse-radish peroxidase(HRP) are widely used in detection of proteins



Types of protein detectio

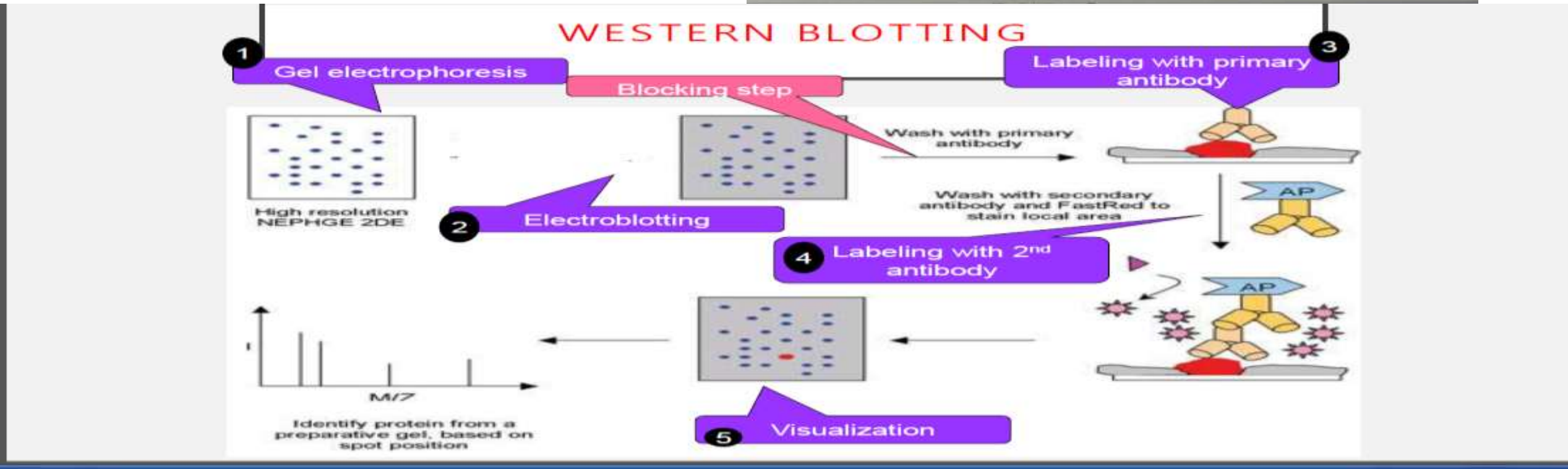
- Chromogenic detection
- Chemiluminescence detection
- Fluorescent detection
- Radioactive detection

INDUSHREE SAHYADRI



Analysis and imaging

This is the last & major step of the western blotting technique.
Detection of signals, using either X-Ray film, scanners or a CCD, results in one or more visible protein bands on the membrane image.



application

Analysis Of IgG Fractions purified from human plasma.
Diagnosis of HIV by ELISA, involves the western blotting technique.
Western blotting technique is also used to Detect Some Forms Of Lyme Disease.
Western blotting technique is used in Definitive Test For BSE, which is commonly know as *Mad cow disease.*
Confirmatory Test For Hepatitis-B involves western blotting technique.
Western blotting test is used in the Analysis Of Biomarkers such as hormones, growth factors & cytokines.
This technique is also employed in The Gene Expression Studies.

Screening methods based on gene expression

1. **Immunological screening**

This involves the use of antibodies that specifically recognize antigenic determinants on the polypeptide. It does not rely upon any particular function of the expressed foreign protein, but requires an antibody specific to the protein.

Earlier immunoscreening methods employed radio-labeled primary antibodies to detect antibody binding to the nitrocellulose sheet (Figure 4-5.3.1(a).). It is now superseded by antibody sandwiches resulting in highly amplified signals. The secondary antibody recognizes the constant region of the primary antibody and is, additionally, conjugated to an easily assayable enzyme (*e.g.* horseradish peroxidase or alkaline phosphatase) which can be assayed using colorimetric change or emission of light using X-ray film.

- In this technique, the cells are grown as colonies on master plates and transferred to a solid matrix.
- These colonies are subjected to lysis releasing the proteins which bind to the matrix.
- These proteins are treated with a primary antibody which specifically binds to the protein (acts as antigen), encoded by the target DNA. The unbound antibodies are removed by washing.
- A secondary antibody is added which specifically binds to the primary antibody removing the unbound antibodies by washing.
- The secondary antibody carries an enzyme label (e.g., horse radishperoxidase or alkaline phosphatase) bound to it which converts colorless substrate to colored product. The colonies with positive results (i.e. colored spots) are identified and subcultured from the master plate.

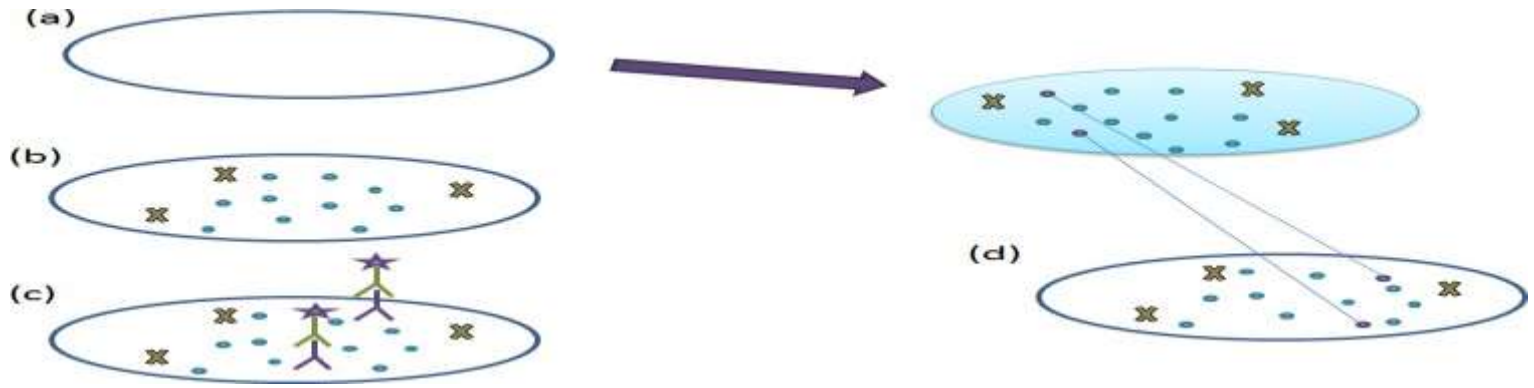


Figure 4-5.3.1(a). Schematic process of immunological screening (a) a nitrocellulose disk is placed onto the surface of an agar plate containing the phage library. Both agar plate and disk are marked so as to realign them later. (b) When the nitrocellulose disk is lifted off again, proteins released from the bacteria by phage lysis bind to the disk. (c) These proteins bind to specific antibody. (d) Plaques formed by bacteriophage that express the protein bound to the antibody will be detected by emission of light. The positive clones can be identified by realignment.

(Adapted from Lodge J. 2007.Gene cloning: principles and applications. Taylor & Francis Group)

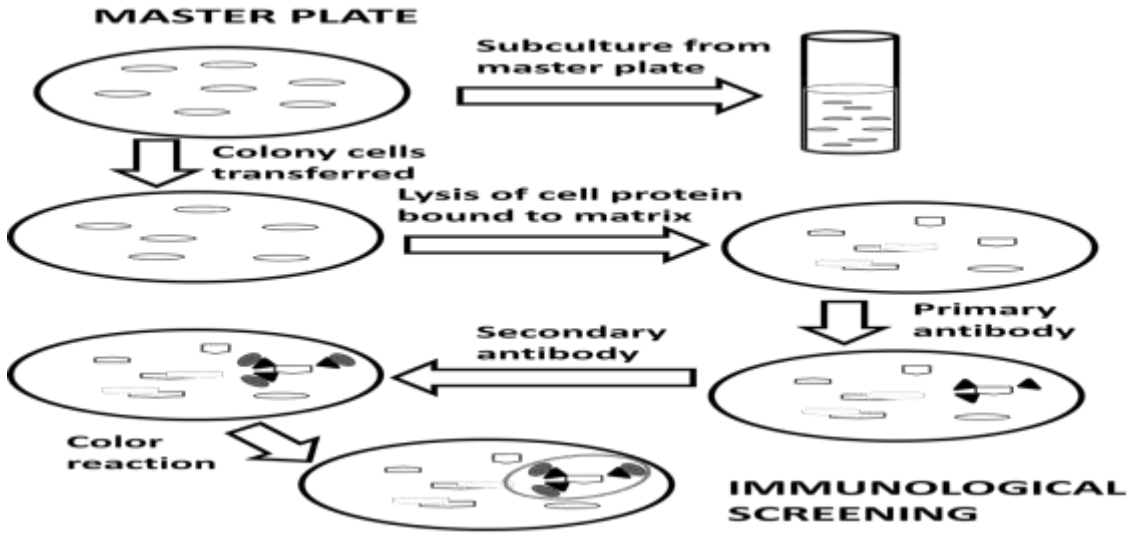


Figure 4-5.3.1(b). Schematic process of immunological screening using antibody sandwich.

The main difficulty with antibody-based screening is to raise a specific antibody for each protein to be detected by injecting a foreign protein or peptide into an animal. This is a lengthy and costly procedure and can only be carried out successfully with proteins produced in reasonably large amounts.

4-5.3.2. Screening by functional complementation

Functional complementation is the process of compensating a missing function in a mutant cell by a particular DNA sequence for restoring the wild-type phenotype. If the mutant cells are non-viable, the cells carrying the clone of interest can be positively selected and isolated. It is a very powerful method of expression cloning and also useful for identification of genes from an organism having same role as that of defective gene in another organism. The selection and identification of positive clones is based on either the gain of function or a visible change in phenotype.

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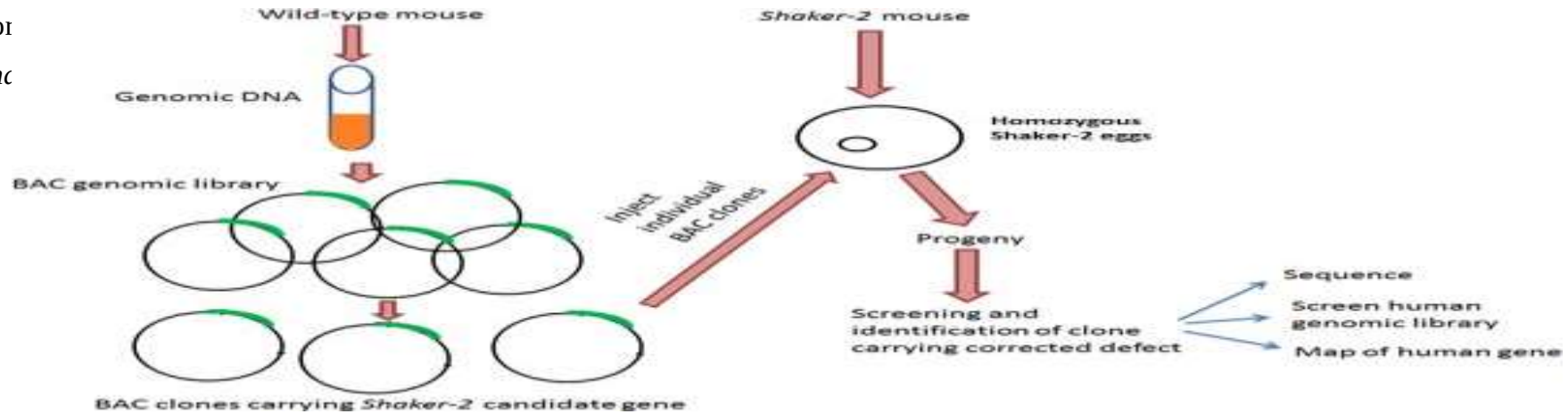


Figure 4-5.3.2. Functional complementation in transgenic mice for isolation of *Shaker-2* gene.

(Adapted from Primrose SB, Twyman RM. 2006. Principles of gene manipulation and genomics.7th ed. Blackwell Publishing.)

The *Shaker-2* mutation is due to the defective gene associated with human deafness disorder. The BAC clone from the wild type mice are prepared and injected into the eggs of Shaker-2 mutants. The resulting mice are then screened for the presence of wild type phenotype. Thus the BAC clone carrying the functional *Shaker-2* gene is identified which encodes a cytoskeletal myosin protein. This method can be used for screening human genomic libraries to identify equivalent human gene.

Drawbacks

- Presence of an assayable mutation within the host cell that can be compensated by the foreign gene expression which in most cases is not available. In addition, foreign genes may not fully compensate the mutations.

Applications

- This method can be used for the isolation of higher-eukaryotic genes (e.g. Drosophila topoisomerase II gene, a number of human RNA polymerase II transcription factors) from an organism.
- It can also be possible in transgenic animals and plants to clone a specific gene from its functional homologue.

Protein structure/function-dependent screening

Besides nucleic acid sequences, the structure/function of its expressed product can also be analyzed

Screening the product of a clone applies only to expression libraries

The clone can be identified because its product is recognized by an antibody or ligand or because the biological activity of the protein is preserved and can be assayed

•For this method the genomic DNA or cDNA library should be constructed in an expression vector. The desired clone is identified on the basis of the structure/function of the protein produced.

•The protein product is separated by lysing the cells of clones and subjecting the cell lysates to polyacrylamide gel electrophoresis. The desired protein on the gel is identified with the help of molecular weight markers.

•To confirm the identified protein, the protein band on the gel is transferred to a nitrocellulose or polyvinylidene membrane. This technique is called western blotting. The desired protein product on the membrane can be confirmed by binding it to a specific antibody/ligand.

Screening by Protein activity

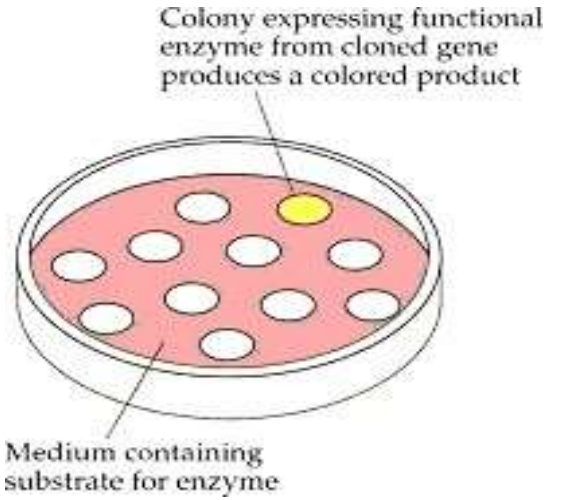
DNA hybridization and immunological assays work well for many kinds of genes and gene products.

1. If the target gene produces an enzyme that is not normally made by the host cell, a direct (in situ) plate assay can be devised to identify members of a library that carry the particular gene encoding that enzyme.

2. The genes for α-amylase, endoglucanase, β-glucosidase, and many other enzymes from various organisms have been isolated in this way.

3. This approach has proven effective for isolating genes encoding biotechnologically useful enzymes from microorganisms present in environmental samples.

4. This technique has enabled the isolation of many novel proteins with interesting properties without the need to first culture the natural host microorganism.



Screening a genomic library for enzyme activity.

1. Cells of a genomic library are plated onto solid medium containing the substrate for the enzyme of interest.
2. If a functional enzyme is produced by a colony that carries a cloned gene encoding the enzyme, the substrate is converted to a colored product that can be easily detected.
3. Note that other, noncolored colonies on the medium also contain fragments of the genomic library, but they do not carry the gene for the enzyme of interest.