TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

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E –content

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BLOTTING TECHNIQUES

- Southern Blotting (For DNA)
- Northern Blotting (For RNA)
- Western Blotting (For Protein)
- Dot blotting (For DNA/RNA)

Blotting Techniques

Blotting techniques are very widely used analytical tools for the specific identification of desired DNA or RNA fragments from thousands of molecules.

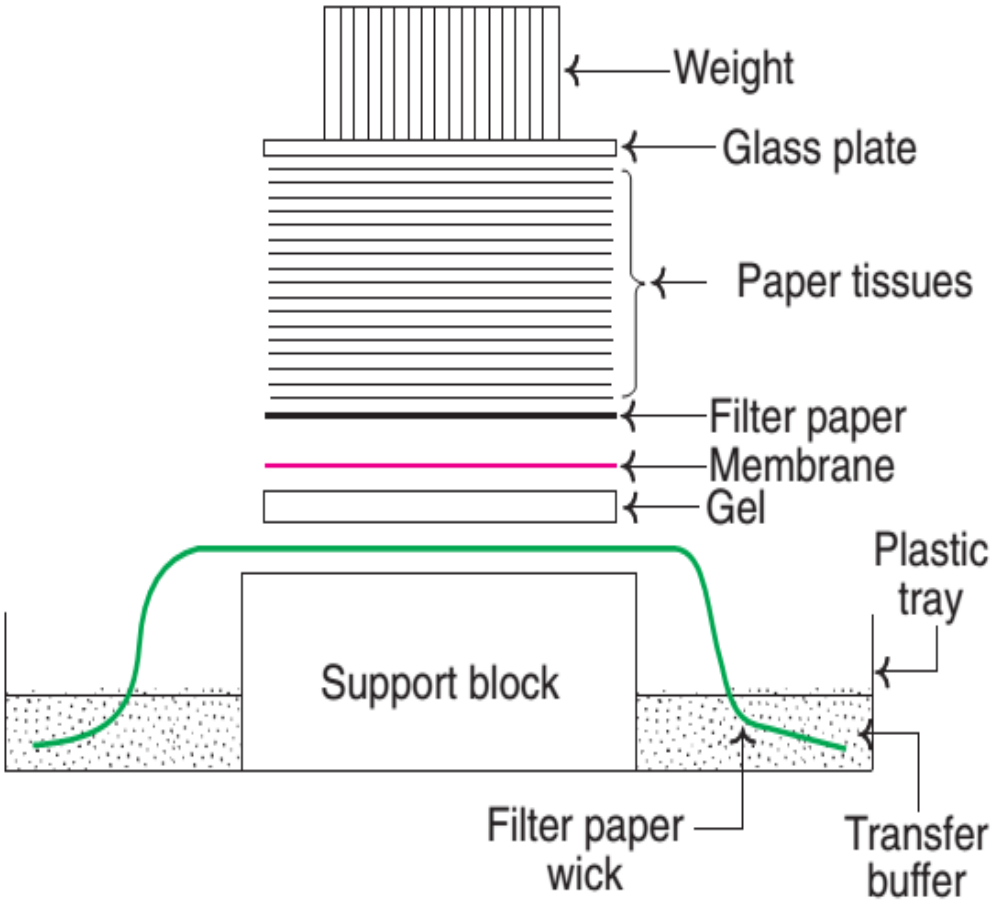
Blotting refers to the process of immobilization of sample nucleic acids on solid support (nitrocellulose or nylon membranes). The blotted nucleic acids are then used as targets in the hybridization experiments for their specific detection.

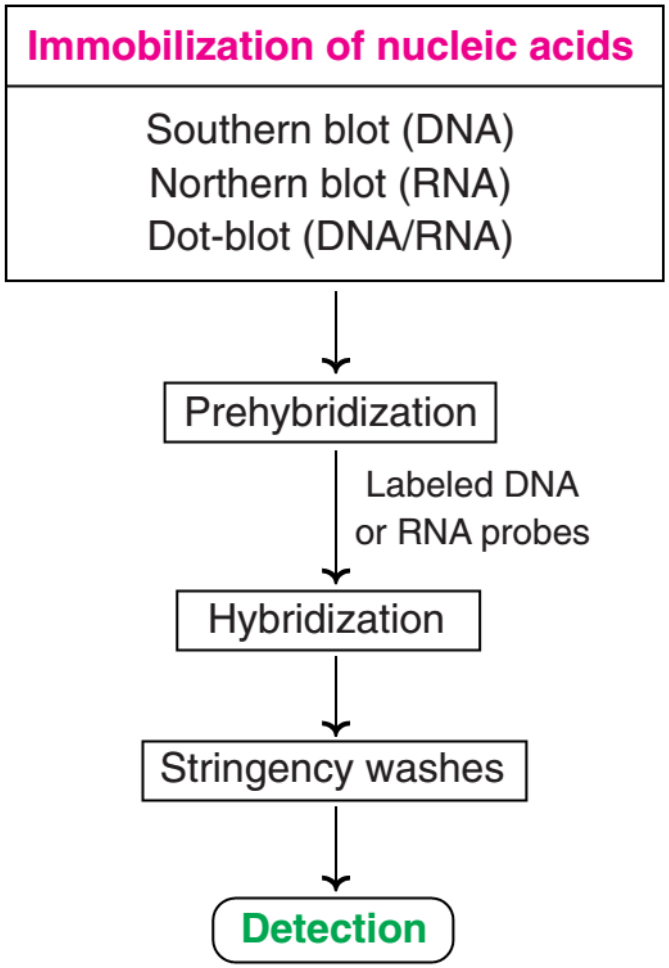
The most commonly used blotting techniques are listed below –

1. Southern blotting (for DNA)
2. Northern blotting (for RNA)
3. Western blotting (for Protein)
4. Dot blotting (DNA/RNA)

The Southern blotting is named after the scientist Ed Southern (1975) who developed it. The other names Northern blotting and Western blotting are laboratory jargons which are now accepted.

Western blotting involves the transfer of protein blots and their identification by using specific antibodies.





Diagrammatic representation of a typical blotting apparatus.
An outline of the nucleic acid blotting techniques.

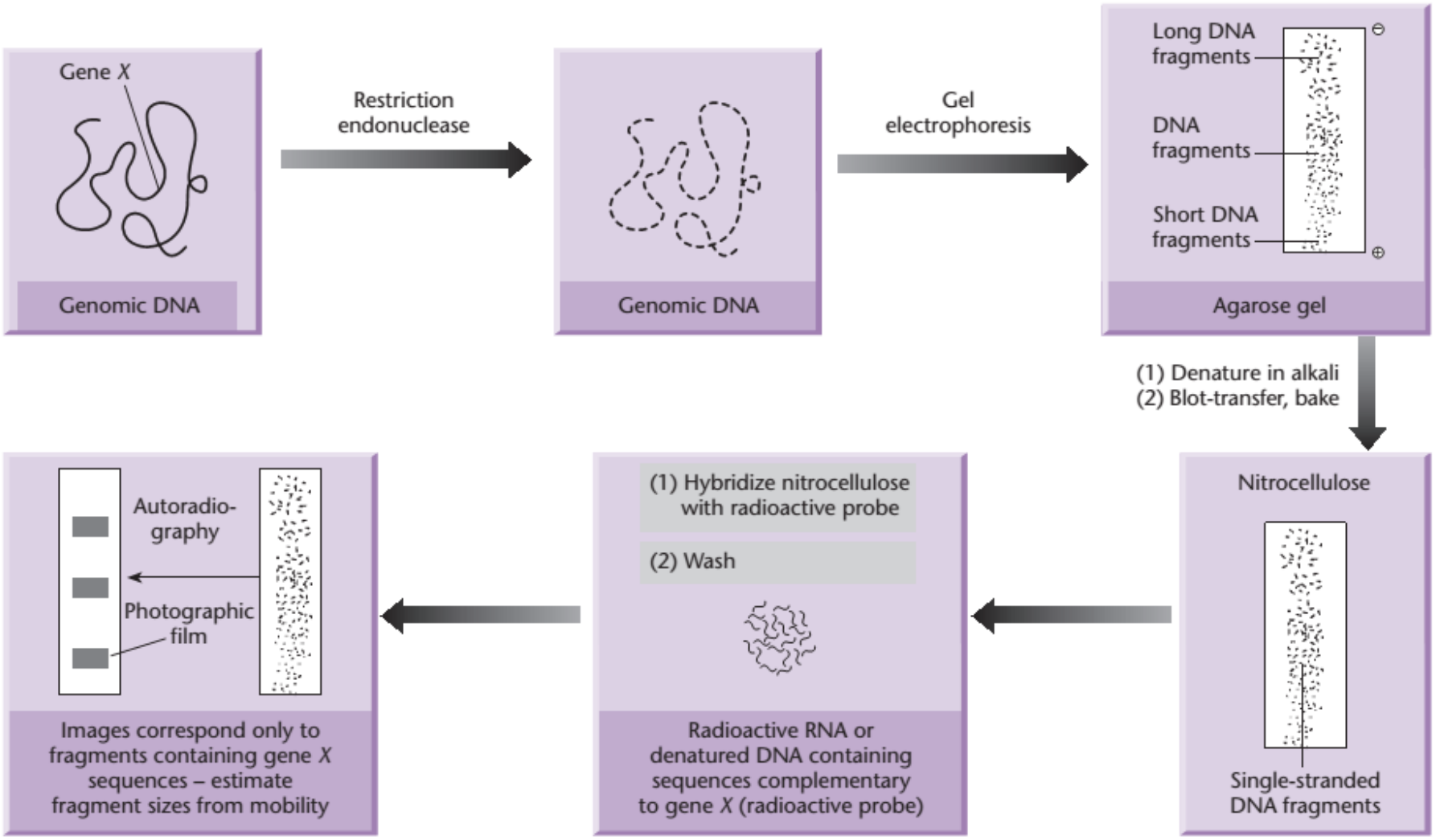
SOUTHERN BLOTTING

Southern blotting technique is the first nucleic acid blotting procedure developed in 1975 by Southern. The genomic DNA isolated from cells/tissues is digested with one or more restriction enzymes. This mixture is loaded into a well in an agarose or polyacrylamide gel and then subjected to electrophoresis.

DNA, being negatively charged migrates towards the anode (positively charged electrode); smaller DNA fragments move faster. The separated DNA molecules are denatured by exposure to a mild alkali and transferred to nitrocellulose or nylon paper. This results in an exact replica of the pattern of DNA fragments on the gel. The DNA can be annealed to the paper on exposure to heat (80°C).

The hybridization membrane is sandwiched between the gel and a stack of paper towels (absorbent material), draw the transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by the buffer flow and immobilized on the membrane. Initially, the membrane material used was nitrocellulose. The main drawback with this membrane is its fragile nature. But nylon in place of nitrocellulose membranes have greater binding capacity for nucleic acids with also high tensile strength.

The nitrocellulose or nylon paper is then exposed to labeled cDNA probes. These probes hybridize with complementary DNA molecules on the paper. The paper after thorough washing is exposed to X-ray film to develop autoradiograph. This reveals specific bands corresponding to the DNA fragments recognized by cDNA probe.



Mapping restriction sites around a hypothetical gene sequence in total genomic DNA by the Southern blot method. Genomic DNA is cleaved with a restriction endonuclease into hundreds of thousands of fragments of various sizes. The fragments are separated according to size by gel electrophoresis and blot-transferred on to nitrocellulose paper. Highly radioactive RNA or denatured DNA complementary in sequence to gene X is applied to the nitrocellulose paper bearing the blotted DNA. The radiolabeled RNA or DNA will hybridize with gene X sequences and can be detected subsequently by autoradiography, so enabling the sizes of restriction fragments containing gene X sequences to be estimated from their electrophoretic mobility. By using several restriction endonucleases singly and in combination, a map of restriction sites in and around gene X can be built up

EFFICIENT SOUTHERN BLOTTING

1. Gel Pretreatment: Large DNA fragments (>10 kb) require a longer transfer time than short fragments. To allow uniform transfer of a wide range of DNA fragment sizes, the electrophoresed DNA is exposed to a short depurination treatment (0.25 mol/l HCl) followed by alkali. This shortens the DNA fragments by alkaline hydrolysis at depurinated sites. It also denatures the fragments prior to transfer, ensuring that they are in the single-stranded state and accessible for probing. Finally, the gel is equilibrated in neutralizing solution prior to blotting. An alternative method uses positively charged nylon membranes, which remove the need for extended gel pretreatment. In this case, the DNA is transferred in native (nondenatured) form and then alkali-denatured in situon the membrane.

2. DAN Fixation on membrane: After transfer, the nucleic acid needs to be fixed to the membrane and a number of methods are available. Oven baking at 80°C is the recommended method for nitrocellulose membranes and this can also be used with nylon membranes. Due to the flammable nature of nitrocellulose, it is important that it is baked in a vacuum oven. An alternative fixation method utilizes ultraviolet cross-linking. It is based on the formation of cross-links between a small fraction of the thymine residues in the DNA and positively charged amino groups on the surface of nylon membranes. A calibration experiment must be performed to determine the optimal fixation period.

3. Hybridization & Autoradiography: Following the fixation step, the membrane is placed in a solution of labeled (radioactive or nonradioactive) RNA, single-stranded DNA, or oligodeoxynucleotide which is complementary in sequence to the blot-transferred DNA band or bands to be detected. Conditions are chosen so that the labeled nucleic acid hybridizes with the DNA on the membrane. Since this labeled nucleic acid is used to detect and locate the complementary sequence, it is called the probe. Conditions are chosen which maximize the rate of hybridization, compatible with a low background of non-specific binding on the membrane.

After the hybridization reaction has been carried out, the membrane is washed to remove unbound radioactivity and regions of hybridization are detected autoradiographically by placing the membrane in contact with X-ray film. A common approach is to carry out the hybridization under conditions of relatively low stringency which permit a high rate of hybridization, followed by a series of post-hybridization washes of increasing stringency (i.e. higher temperature or, more commonly, lower ionic strength). Autoradiography following each washing stage will reveal any DNA bands that are related to, but not perfectly complementary with, the probe and will also permit an estimate of the degree of mismatching to be made.

Applications of Southern blotting

1. The Southern blotting methodology can be extremely sensitive.
2. It can be applied to mapping restriction sites around a single-copy gene sequence in a complex genome such as that of humans.
3. It is an invaluable method in gene analysis.
4. Important for confirmation of DNA cloning.
5. Forensically applied to detect minute quantities of DNA (to identify parenthood, thieves, rapists etc.)
6. when a “mini-satellite” probe is used it can be applied forensically to minute amounts of DNA.

NORTHERN BLOTTING

Northern blotting is a variant of Southern blotting that is used for RNA analysis. Southern's technique was thought that it could not be applied directly to the blot-transfer of RNAs separated by gel electrophoresis, since RNA was found not to bind to nitrocellulose.

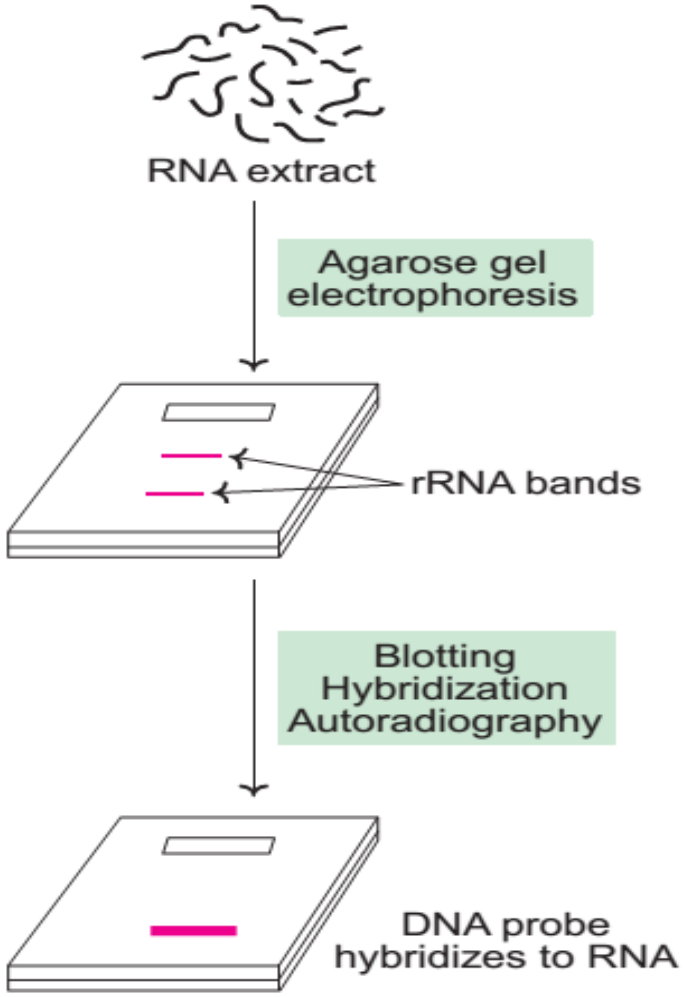
Alwine et al. (1979) therefore devised a procedure in which RNA bands are blot transferred from the gel on to chemically reactive paper, where they are bound covalently. The reactive paper is prepared by diazotization of aminobenzyloxymethyl paper (creating diazobenzyloxymethyl (DBM) paper), which itself can be prepared from Whatman 540 paper by a series of uncomplicated reactions. Once covalently bound, the RNA is available for hybridization with radiolabeled DNA probes. As before, hybridizing bands are located by autoradiography. Alwine et al.'s method thus extends that of Southern and for this reason it has acquired the jargon term northern blotting.

Subsequently it was found that RNA bands can indeed be blotted on to nitrocellulose membranes under appropriate conditions (Thomas 1980) and
suitable nylon membranes have been developed. Because of the convenience of these more recent methods, which do not require freshly activated paper, the use of DBM paper has been superseded.

Northern blotting is the technique for the specific identification of RNA molecules. The procedure adopted is almost similar to that described for Southern blotting.

RNA molecules are subjected to electrophoresis, followed by blot transfer, hybridization and autoradiography. RNA molecules do not easily bind to
nitrocellulose paper or nylon membranes. Blot transfer of RNA molecules is carried out by using a chemically reactive paper prepared by diazotization of aminobenzyloxymethyl to create diazobenzyloxymethyl (DBM) paper. The RNA can covalently bind to DBM paper.

Northern blotting is theoretically, a good technique for determining the number of genes (through mRNA) present on a given DNA. But this is not really practicable since each gene may give rise to two or more RNA transcripts. Another drawback is the presence of exons and introns



An outline of Northern blotting

WESTERN BLOTTING/ PROTEIN IMMUNOBLOTTING

Western blotting is used to transfer proteins from acrylamide gels to membranes. The term “western” blotting was coined by Burnette 1981.

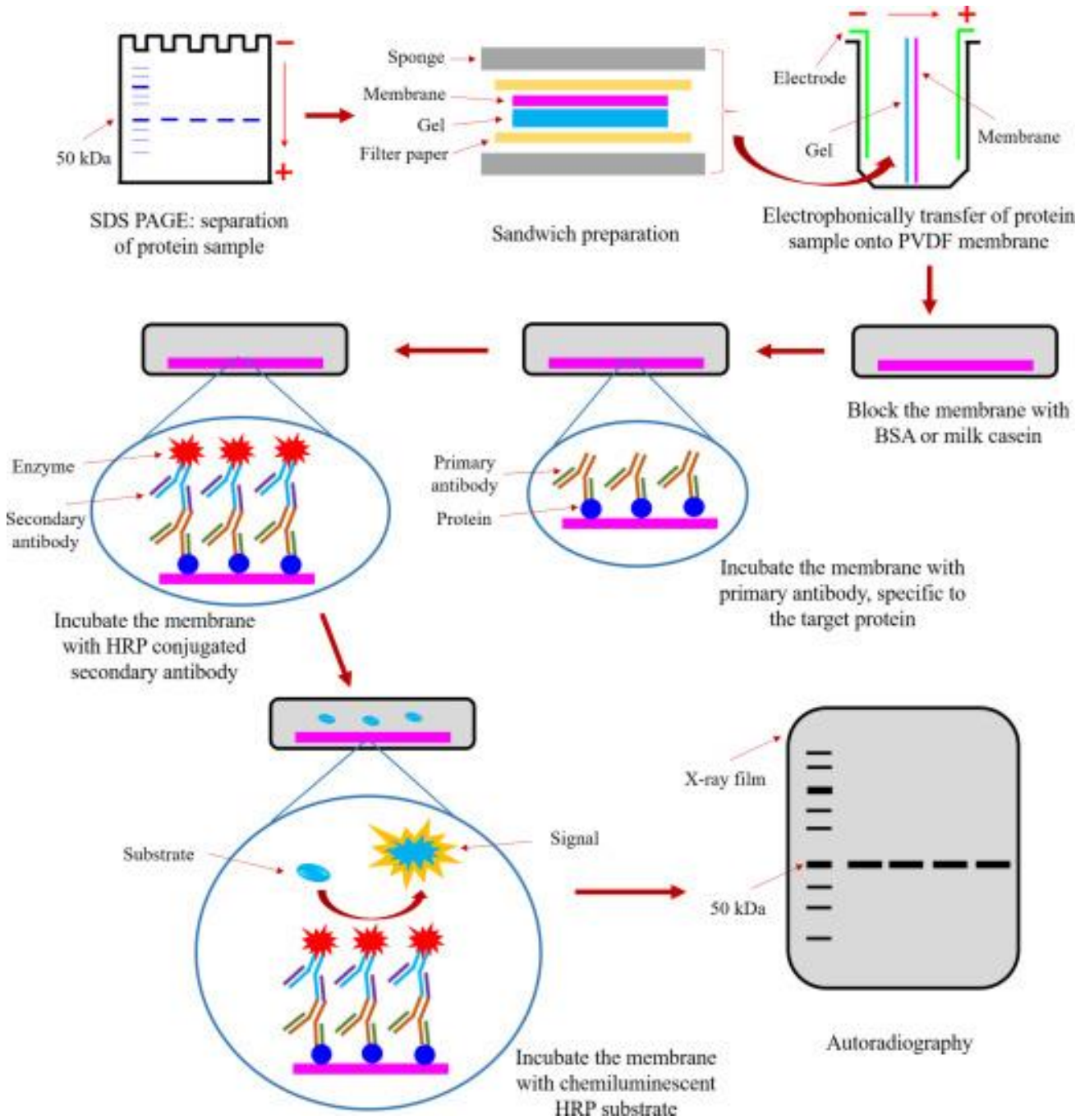
It involves the transfer of electrophoresed protein bands from a polyacrylamide gel on to a membrane of nitrocellulose or nylon, to which they bind strongly (Gershoni & Palade 1982, Renart & Sandoval 1984). The bound proteins are then available for analysis by a variety of specific protein–ligand interactions.

Most commonly, antibodies are used to detect specific antigens. Lectins have been used to identify glycoproteins.

In these cases the probe may itself be labeled with radioactivity, or some other “tag” may be employed. Often, however, the probe is unlabeled and is itself detected in a “sandwich” reaction, using a second molecule which is labeled, for instance a species-specific second antibody, or protein A of Staphylococcus aureus (which binds to certain subclasses of IgG antibodies), or streptavidin (which binds to antibody probes that have been biotinylated). These second molecules may be labeled in a variety of ways with radioactive, enzyme, or fluorescent tags.

An advantage of the sandwich approach is that a single preparation of labeled second molecule can be employed as a general detector for different probes. For example, an antiserum may be raised in rabbits which reacts with a range of mouse immunoglobins. Such a rabbit anti-mouse (RAM) antiserum may be radio-labeled and used in a number of different applications to identify polypeptide bands probed with different, specific, monoclonal antibodies, each monoclonal antibody being of mouse origin.

The sandwich method may also give a substantial increase in sensitivity, owing to the multivalent binding of antibody molecules.



DOT-BLOTTING

- Dot-blotting is a modification of Southern and Northern blotting techniques described above. In this approach, the nucleic acids (DNA or RNA) are directly spotted onto the filters, and not subjected to electrophoresis. The hybridization procedure is the same as in original blotting techniques.

- Dot-blotting technique is particularly useful in obtaining quantitative data for the evaluation of gene expression.

VACUUM DRIVEN BLOTTING

- Vacuum Blotting (Olszewska & Jones 1988), has several advantages over capillary or electrophoretic transfer methods: transfer is very rapid and gel treatment can be performed in situ on the vacuum apparatus.

- This ensures minimal gel handling and, together with the rapid transfer, prevents significant DNA diffusion

AUTORADIOGRAPHY IN BLOTTING

Principle

The localization and recording of a radiolabel within a solid specimen is known as autoradiography and involves the production of an image in a photographic emulsion. Such emulsions consist of silver halide crystals suspended in a clear phase composed mainly of gelatin.

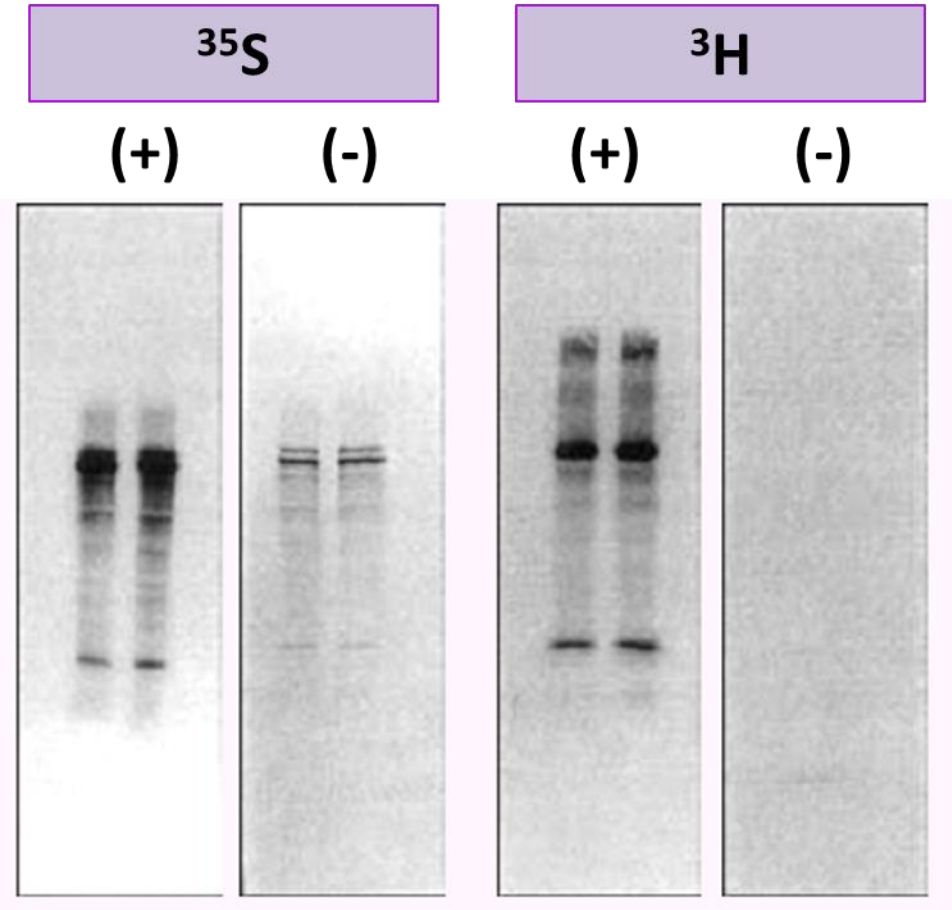
When a b-particle or g-ray from a radionuclide passes through the emulsion, the silver ions are converted to silver atoms. This results in a latent image being produced, which is converted to a visible image when the image is developed.

Development is a system of amplification in which the silver atoms cause the entire silver halide crystal to be reduced to metallic silver. Unexposed crystals are removed by dissolution in fixer, giving an autoradiographic image which represents the distribution of radiolabel in the original sample

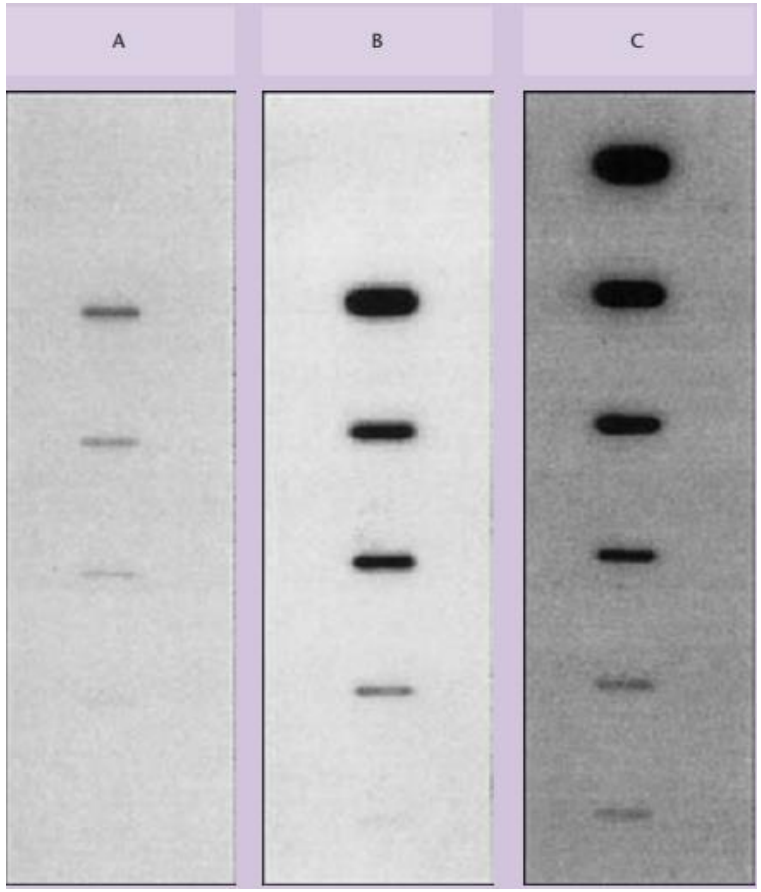
Procedure

In **Direct Autoradiography**, the sample is placed in intimate contact with the film and the radioactive emissions produce black areas on the developed autoradiograph. It is bestsuited to detection of weak- to mediumstrength b-emitting radionuclides (^{3}H, ^{14}C, ^{35}S). Direct autoradiography is not suited to the detection of highly energetic b-particles, such as those from ^{32}P, or for g-rays emitted from isotopes like ^{125}I. These emissions pass through and beyond the film, with the majority of the energy being wasted. Both ^{32}P and ^{125}I are best detected by indirect autoradiography.

Indirect Autoradiography describes the technique by which emitted energy is converted to light by means of a scintillator, using fluorography or intensifying screens. In fluorography the sample is impregnated with a liquid scintillator. The radioactive emissions transfer their energy to the scintillator molecules, which then emit photons which expose the photographic emulsion. Fluorography is mostly used to improve the detection of weak b-emitters. Intensifying screens are sheets of a solid inorganic scintillator which are placed behind the film. Any emissions passing through the photographic emulsion are absorbed by the screen and converted to light, effectively superimposing a photographic image upon the direct autoradiographic image.



Autoradiographs showing the detection of ^{35}S- and ^{3}H-labeled proteins in acrylamide gels with (+) and without (−) fluorography.



The improvement in sensitivity of detection of ^{125}I-labeled IgG by autoradiography obtained by using an intensifying screen and pre-flashed film. A, no screen and no pre-flashing; B, screen present but film not pre-flashed; C, use of screen and pre-flashed film.

SENSITIVITY

- The gain in sensitivity which is achieved by use of indirect autoradiography is offset by nonlinearity of film response.

- A single hit by a b-particle or g-ray can produce hundreds of silver atoms, but a single hit by a photon of light produces only a single silver atom. Although two or more silver atoms in a silver halide crystal are stable, a single silver atom is unstable and reverts to a silver ion very rapidly. This means that the probability of a second photon being captured before the first silver atom has reverted is greater for large amounts of radioactivity than for small amounts. Hence small amounts of radioactivity are underrepresented with the use of fluorography and intensifying screens. This problem can be overcome by a combination of pre-exposing a film to an instantaneous flash of light (preflashing) and exposing the autoradiograph at −70°C.

- Pre-flashing provides many of the silver halide crystals of the film with a stable pair of silver atoms. Lowering the temperature to −70°C increases the stability of a single silver atom, increasing the time available to capture a second photon

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