# Molecular Biology: Blotting / Hybridization techniques

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INTRODUCTION

Blotting is a common laboratory procedure in which biological molecules in a gel matrix are transferred onto nitrocellulose or nylon membrane for further scientific analysis. The biological molecules transferred in this process are DNA, RNA or proteins. The blotting procedure is named differently depending on the type of the molecules being transferred. When DNA fragments are transferred the procedure is called a Southern blot, named after Edward Southern that first developed it. The Northern blotting procedure, which transfers RNA molecules, was developed shortly thereafter and humorous named Northern blotting. Western blotting involves the transfer of proteins.

All blotting procedures begin with a standard process called gel electrophoresis when DNA, RNA, or proteins are loaded on to an agarose or acrylamide gel and separated on the gel through an electric field. Two types of gels are commonly used: agarose gels and acrylamide gels. Transfer is initiated when nitrocellulose or nylon membrane is laid on top of the gel and biological molecules are transfer from the gel to the membrane. Hybridization / blotting is a technique in which biological molecular (DNA, RNA or protein) are immobilized onto a nylon or nitrocellulose membrane. A probe (a piece of nucleic acid with identical and specific sequence to the organism or gene of interest) can then hybridize (join) to the biological molecules (DNA, RNA or protein) with identical sequence on the membrane. The hybridization between the blotted DNA and probe is visualized by labeling the probe in some way. Short fragments of DNA that have a nucleotide sequence complementary to the molecule being analyzed are normally used as probes in Southern and Northern blots. Antibodies that react with the protein being analyzed are used as probes in a Western blot.

BLOTTING

Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support, generally nylon or nitrocellulose membranes. There are different blotting procedures depending on the type of molecule being transferred. When DNA fragments are transferred the procedure is called a Southern blot, named for the man who first developed it, Edward Southern. With Northern blotting, RNA molecules are transferred and with Western blotting, protein molecules are transferred.

Southern blotting

The Southern blot is use to verify the presence or absence of a specific nucleotide sequence in DNA from different sources. DNA is isolated from each source and then digested with a specific restriction enzyme. The DNA restriction fragments are loaded onto an agarose gel and the fragments separated by electrophoresis according to size, with the smaller fragments migrating faster than the larger fragments. The fragments are made visible with ethidium bromide under UV-light (ethidium bromide fluoresces under UV light when intercalated into the major groove of DNA or RNA). The DNA is then transferred from the agarose gel to a membrane (nylon or nitrocellulose) and denatured to produce single strands. A nucleic acid probe is labeled, usually by incorporating radioactivity or tagging the molecule with a fluorescent dye. The labeled probe is added and it binds to complementary DNA on
the membrane. To detect the position of the labeled probe, the membrane is covered with an X-ray film and after development the position of the probe becomes visible (Figure 1).

**Figure 1: Schematic representation of a Southern blotting: transfer of separated DNA fragments to a membrane and hybridization to a labeled probe**

Northern blotting

Northern blotting is used to study gene expression by analyzing the RNA of an organism. RNA is isolated from a sample, separated by electrophoresis, immobilized and detected by either a RNA or DNA probe.
**Dot-blotting:**

Dot-blot hybridization is a technique where the genome segments of the organisms are not separated (no electrophoresis step), but DNA/RNA is blotted on a membrane.

**Western blotting:**

A similar principle to DNA hybridization can be used to detect proteins, using Antigen (Ag)-Antibody (Ab) binding. In Western blotting, protein/antigens are transferred to a membrane and probed with labeled proteins/antibodies. The technique is therefore sometimes called the protein immunoblot. Protein dot-blot assays are also available, although they are not very sensitive and are not often used as a diagnostic tool.

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**FIXATION OF NUCLEIC ACIDS ONTO MEMBRANES**

A range of membranes is now available for hybridization experiments. Originally, nitrocellulose was the most widely used as it provides high sensitivity and low background signals. However, the various newer nylon-based membranes are physically much stronger. Single stranded DNA (ssDNA) is immobilized on nitrocellulose membranes under conditions of high salt (>1 M NaCl), and must be heated at 80 °C in a vacuum to irreversibly attach the nucleic acid. Nylon membranes bind all nucleic acids under a wide range of salt concentrations, and irreversible or covalent attachment can be achieved by UV irradiation for 5 min or less, or by treatment with 0.4 M NaOH. Similarly DNA of bacterial colonies or bacteriophage plaques grown up on solid medium plates can be transferred to membranes to enable a single colony or plaque of interest to be identified amongst large numbers of irrelevant ones (as in a gene library). The DNA on the membrane is hybridized to a specific probe and the colonies identified can then be picked off the original master plate for further processing.

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**LABELING METHODS**

Classically, probes were radioactively labeled, often with $^{32}\text{P}$ because of its high energy and ease of incorporation into the phosphate groups of dNTPs. Radioactively labeled probes are detected using X-ray film. Disadvantages of $^{32}\text{P}$ are its short half-life (about 2 weeks), contamination problems (all materials and equipment must be dedicated to radioactive work only), and expense of disposal of radioactive waste.

Many different non-radioactive detection systems are available, depending on the label used. Broadly, the categories of non-radioactive detection are colourimetric, fluorescent, and chemiluminescent.

**Colourimetric** detection generally involves the production of a coloured precipitate which can be seen with the naked eye. In a typical system, the DNA probe itself is labeled with an antigen such as digoxigenin; following hybridization to its target it would be exposed to an anti-digoxigenin antibody conjugated to an enzyme capable of catalyzing a colourimetric reaction.
**Fluorescent** detection involves probes which are directly labeled with fluorophores, or more likely, probes which are coupled to fluorescent molecules indirectly. For example, if a probe is labeled with biotin, it would be exposed to avidin conjugated to a fluorescent tag (avidin is a protein which contains four identical subunits, each of which can bind to biotin with a high degree of affinity and specificity). Fluorophores emit light when excited by light of an appropriate wavelength; the emitted light can then be detected.

**Chemiluminescence** is the result of a chemical or enzymatic reaction that triggers the release of ordinary visible light which can then be detected. Examples include firefly luciferase and enhanced chemiluminescence (ECL) using horseradish peroxidase enzyme (HRP). In a luciferase reaction, light is emitted when luciferase acts on the appropriate luciferin substrate. HRP catalyzes the conversion of enhanced chemiluminescent substrate into a sensitized reagent, which, on further oxidation by hydrogen peroxide, produces a triplet carbonyl group which emits light when it decays to the singlet carbonyl.

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**LABELING OF DNA PROBES**

The label (radioactive or non-radioactive) is attached to one of the four nucleotides (dNTPs) and then incorporated into the probe using one of the following methods:

**PCR labeling** is a straightforward and now very popular method of incorporating labeled nucleotides into a probe. Primers are designed to amplify the desired probe sequence, and a labeled dNTP is simply included during the PCR.

**Nick translation** involves the action of two enzymes (DNase I and DNA polymerase I) on the double-stranded DNA (dsDNA) to be labeled in the presence of a labeled nucleotide. Under conditions designed to limit its activity, DNase I will randomly introduce a few nicks (i.e. single strand breaks) in the dsDNA backbone. These nicks are repaired by DNA polymerase I. DNA polymerase I synthesizes DNA in a 5’-3’ direction and it also has exonuclease activity. These two activities act together to remove and then replace a few nucleotides down from each nick, incorporating the labeled dNTP in the process. The newly formed bits of DNA are now labeled and can be used as a probe.

**Random oligo primed synthesis** is a technique in which dsDNA is denatured into single strands and annealed to random hexamer oligonucleotides. These random primers can then be extended using DNA polymerase (Klenow), incorporating labeled nucleotides (see Figure 2).
**Figure 2: Random priming for the preparation of a labelled probe**

**End labeling** involves techniques in which one end of a DNA (or RNA) molecule is specifically labeled. The 5’ or 3’ end can be labeled. With 5’ end labeling a terminal phosphate group (usually radioactive $^{32}$P) is donated from a dNTP to the 5’–OH group, and with 3’ ends a small chain of identical labeled nucleotides are added.

**MELTING TEMPERATURES**

Hybridization (procedure where probe and membrane are incubated to allow annealing of probe to immobilized DNA/RNA on membrane) and washes (the probe that did not bind or bound nonspecifically to DNA/RNA on membrane must be washed from the membrane) must be done at specific temperatures. To determine the temperature(s) to be used, the melting temperature ($T_m$) of the probe must be determined. All double-stranded nucleic acids - whether dsDNA, dsRNA or RNA:DNA hybrids - have specific “melting temperatures”, which depend mainly on their specific guanine + cytosine (G + C) content, but also on whether they are DNA, RNA, or a mixture (RNA:RNA hybrids have the highest melting temperatures, followed by DNA:RNA hybrids, then dsDNA), and on the ionic strength of the solution.
The $T_m$ is also dependent on the length of the sequences to be annealed: the shorter the probe sequence, the lower the melting temperature. The degree of sequence mismatch also determines the effective melting temperature of a hybrid: $T_m$ decreases by about 1 °C for every 1 % of mismatched base pairs. It therefore makes sense to maximize probe length in order to minimize $T_m$ reduction due both to length and degree of sequence mismatch. Under standard conditions of annealing (0.8 M NaCl, neutral pH) one may calculate the melting temperature ($T_m$) of any given DNA hybrid as shown:

$$T_m = 81.5\ °C + 0.41(\%G + \%C) - 550/n$$

where $n$ = probe length (number of nucleotides).

One can see that the reduction in $T_m$ becomes negligible for probes of length 200 nucleotides or greater. Thus, one may vary the specificity of association of a specific single-stranded "probe" and a target by varying the incubation temperature of the annealing reaction: the higher the temperature, the higher the specificity of the reaction - and the lower the likelihood of annealing taking place.

**STRINGENCY AND WASHING PROCEDURE**

The successful use of nucleic acids as probes for detecting sequences of interest therefore depends on certain reaction conditions, which are in turn determined by the physical properties (i.e. length and sequence) of the probe. This leads to the concept of stringency of hybridization: one increases the stringency by lessening the likelihood of non-homologous annealing. This can be done by simply increasing the temperature of incubation - bearing in mind that rate of hybridization/annealing is maximal at about $T_m - 25\°C$, and too high a temperature results in very slow annealing. An acceptable compromise is to anneal at a standard temperature (e.g. 65 °C), and then to wash the annealed and immobilized hybrid molecules to varying degrees of stringency: the extent to which one should wash can be assessed by repeated autoradiography if the probe is $^{32}P$-labeled, or by repeated colour assay of replicates in the case of non-radioactively labeled probes. Washing stringency may be increased by varying the ionic strength (from 1.0 M NaCl to 0.02 M), or varying the temperature (ambient to 65 °C) of the buffer system. One may also include SDS or another detergent in the wash and hybridization buffers in order to decrease non-specific attachment of the probe to the adsorptive membrane. For this reason a blocking or prehybridization buffer is generally used before and during the annealing reaction, to block adsorptive sites on the membrane not occupied by target nucleic acid. This usually consists of buffer salts, detergent, protein, inert polymer material, and DNA.
WESTERN BLOTTING PROCEDURE

The reason for transferring proteins to membranes from gels is to be able to get at them more efficiently with various probes, as polyacrylamide is not particularly amenable to the diffusion of large molecules. The most popular type of probe of immobilized proteins is an antibody of one type or another: the attachment of specific antibodies to specific immobilized antigens can be readily visualized by indirect enzyme immunoassay techniques, usually using a chromogenic substrate, which produces an insoluble product. Lately chemiluminescent substrates have begun to be used because of their greater detection sensitivity. Other possibilities for probing include the use of fluorescent or radioisotope labels (e.g. fluorescein, ^{125}\text{I})\): Probes for the detection of antibody binding can be conjugated anti-immunoglobulins (e.g. goat-anti-rabbit / human); conjugated staphylococcal Protein A (which binds IgG of various species of animal); or probes to biotinylated / digoxigeninylated primary antibodies (e.g. conjugated avidin / streptavidin / antibody).

The immunoassay is usually done by blocking the transfer membrane with a concentrated protein solution (e.g. 10 % foetal calf serum, 5 % non-fat milk powder) to prevent further non-specific binding of proteins; this is followed by incubation of the membrane in a diluted antiserum / antibody solution, washing of the membrane, incubation in diluted conjugated probe antibody or other detecting reagent, further washing, and the colourimetric / autoradiographic / chemiluminescent detection.

The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass: proteins are first separated by mass in the SDS-PAGE, then specifically detected in the immunoassay step.

APPLICATIONS OF BLOTTING

The use of probes for detection of genes and diagnosis of disease

Nucleic acid hybridization can be used to identify a particular recombinant clone if a DNA or RNA probe, complementary to the desired gene, is available.

It can therefore be used to:

- identify the correct clone after gene cloning
- identify and diagnose specific nucleic acids in a sample
- identify specific nucleic acids in tissue sections and assist in the study of pathogenesis

Nucleic acid hybridization assays with gene probes have been used to detect a variety of mutations that produce single-gene disorders, such as the prenatal diagnosis of sickle cell anemia, alpha-thalassemia, and beta-thalassemia. However, many of these tests have been superseded by PCR and real-time PCR diagnostic assays.
**In situ hybridization**

The technique of *in situ* hybridization is used to locate nucleic acid inside a specific cell using either DNA or RNA as a probe. The theory is the same as for Southern hybridization, except that the DNA to which the probe will hybridize is not on a membrane but within the cell.

**Selecting gene or region by hybridization**

Nucleic acid hybridization can be used to screen libraries where the genome of an organism has been fragmented and cloned into a vector. A probe is used to screen the genomic library to select a clone containing a specific gene / region homologous to the probe.

**Immunoassays**

Western blotting can be used to determine in which tissues, or under which physiological conditions a gene is expressed to produce a protein. It can be used to determine the immunodominant proteins in a pathogenic organism, by screening size-separated proteins of the pathogen with antisera from an infected host. Such immunodominant proteins might be important in protection from infection and could be used as components of a recombinant vaccine.

A Western blot is used as the confirmatory test for HIV. This test detects anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted onto a membrane. The human serum sample to be tested is applied and if anti-HIV antibody is present, it binds to the HIV antigens on the membrane. During the washing steps, unbound antibodies are washed away. A secondary anti-human antibody linked to an enzyme signal is added. Any stained bands indicate the proteins to which the patient's serum contains antibody. A similar Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').

It is also possible to use a technique similar to Western blotting to elute specific antibodies from specific proteins resolved out of a complex mixture, many of whose components react with a given antiserum: one can electrophorese a mixture of proteins, cut out a specific band from a gel or membrane, and use this to obtain specific antibodies from a serum.

**Micro-arrays**

Micro-arrays are miniature arrays of gene fragments attached to glass slides. Micro amounts of nucleic acids are blotted onto slides and hybridized with specific probes in a small chamber. This requires very expensive specialized equipment and leads to the identification of gene changes. A robotic instrument is used to print the DNA spots simply by dipping pins into wells containing the DNA and then striking these pins on the surface of the glass slides.

Micro-arrays have spot diameters of <250 microns. DNA arrays are also referred to as DNA chips. Depending on the spotting technique used, the number of spots on a glass micro-array can range from hundreds to thousands. A micro-array permits rapid analysis of thousands of genes for the purposes of gene discovery, sequencing, mapping, expression analysis, and polymorphism detection.
The application of micro-arrays will lead to an improved understanding of the gene changes occurring in human diseases, to the development of new diagnostic tools as well as to more effective drugs. In the basic sciences, micro-array technology permits researchers to elucidate the genetic pathways that are followed during development and aging, as well as pathology of specific cells or tissues of the body.

**HYBRIDIZATION BASED ON AN EXAMPLE: REVERSE LINE BLOT (RLB)**

In a reverse line blot, species-specific probes are immobilized on a membrane and then biotin-labeled PCR products from specific samples are hybridized to the membrane. Peroxidase-labeled streptavidin is added and hybridized PCR products are detected by enhanced chemiluminescence.

**Background:** Ticks transmit a variety of pathogenic microorganisms that include tick-borne protozoa and tick-borne bacteria. There are many tick-borne protozoa belonging to the genera *Theileria* and *Babesia* which infect domestic and wild ruminants, and important tick-borne bacteria include *Anaplasma* species and *Ehrlichia* species. An RLB has been developed which can be used to simultaneously detect common tick-borne pathogens of these four different genera in samples from cattle and small ruminant hosts. Probes consist of sequences unique to *Ehrlichia*, *Anaplasma*, *Theileria* and *Babesia* in this case targeting 16S and 18S ribosomal RNA (rRNA) genes. Blood samples are taken from animals to detect single or multiple infections. DNA isolated from blood samples is used as template to amplify the 16S and 18S rRNA genes using PCR. One of the PCR primers is synthesized with a biotin label, so that the resulting PCR product is biotin-labeled. The PCR products are hybridized to a membrane on which *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species-specific probes are immobilized. Positive and negative controls are included. After stringency washes, peroxidase-labeled streptavidin is added and the hybridized PCR products are visualised by enhanced chemiluminescence. The results enable the researcher to determine if animals have no, single or multiple infections of different pathogens.

**FREQUENTLY ASKED QUESTIONS**

1. **What is hybridization?**

   Hybridization or binding of biological molecular with similar or identical sequences provides information about the molecular identity, size, and abundance.

2. **What is the difference between a Southern Blot hybridization and a Northern Blot hybridization?**

   Southern Blots are used to probe DNA, while Northern Blots are used to probe RNA.
3. **What are the purpose and usefulness of Southern blotting for screening DNA sequences?**

   The purpose of a Southern blot is to determine if a particular DNA sequence is present along with its size. The usefulness of this technique is due to the fact that you can determine whether or not a particular DNA sequence is present in DNA samples from different sources.

4. **What is a northern blot?**

   A technique for identifying specific sequences of RNA in which RNA molecules are separated by electrophoresis, transferred to nitrocellulose, and identified with a suitable probe.

5. **What are the uses of the Northern Blot?**

   Uses of the Northern Blot. Northern analysis is used for detecting and quantifying mRNA.

6. **What is a western blot?**

   Protein blotting is an analytical method that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. In western blotting (or immunoblotting), prior to protein immobilization on the membranes sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) providing information about molecular weight and the potential existence of different isoforms of the proteins under study.

7. **What is the best labeling to use for each blotting target?**

   Best labeling listed with each biological molecule/target
   
   **DNA**
   - Nick translational labeling
   - Random primed DNA labeling
   - PCR labeling of DNA probes
   - Oligonucleotide 3’ / 5’ end labeling
   
   **RNA**
   - Transcriptional labeling of RNA probes
   
   **Oligonucleotides**
   - Oligonucleotide 3’ / 5’ end labeling
   - Oligonucleotide tailing
8. **What is the difference between dot blotting and reverse line blot?**

   With reverse line blot probes are immobilized on the membrane rather than the PCR products, and the PCR products are labeled rather than the probes whereas in dot blots the PCR products are immobilized on the membrane and the probes are labeled.

9. **Is Western blot a quantitative method?**

   No, it is semi-quantitative meaning you cannot determine absolute protein levels but can detect differences between groups.

10. **What is dot blotting?**

    Hybridization technique where biological molecules are not separated using electrophoresis but directly blotted followed by hybridization of biological molecular to the immobilized target on the membrane.

**REFERENCES**

4. [http://openwetware.org/wiki/Protein_blot_%28Western%29](http://openwetware.org/wiki/Protein_blot_%28Western%29)