**A Southern blot** is a method used in [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology) for detection of a specific [DNA sequence](https://en.wikipedia.org/wiki/DNA_sequence) in DNA samples. Southern blotting combines transfer of [electrophoresis](https://en.wikipedia.org/wiki/Agarose_gel_electrophoresis)-separated DNA fragments to a filter membrane and subsequent fragment detection by [probe hybridization](https://en.wikipedia.org/wiki/Probe_hybridization).

The method is named after the [British](https://en.wikipedia.org/wiki/United_Kingdom) [biologist](https://en.wikipedia.org/wiki/Biologist) [Edwin Southern](https://en.wikipedia.org/wiki/Edwin_Southern), who first published it in 1975.[[1]](https://en.wikipedia.org/wiki/Southern_blot#cite_note-1) Other [blotting](https://en.wikipedia.org/wiki/Blot_%28biology%29) methods (i.e., [western blot](https://en.wikipedia.org/wiki/Western_blot),[[2]](https://en.wikipedia.org/wiki/Southern_blot#cite_note-2) [northern blot](https://en.wikipedia.org/wiki/Northern_blot), [eastern blot](https://en.wikipedia.org/wiki/Eastern_blotting), [southwestern blot](https://en.wikipedia.org/wiki/Southwestern_blot)) that employ similar principles, but using RNA or protein, have later been named in reference to Edwin Southern's name. As the label is [eponymous](https://en.wikipedia.org/wiki/Eponym), Southern is capitalised, as is conventional of [proper nouns](https://en.wikipedia.org/wiki/Proper_noun). The names for other blotting methods may follow this convention, by analogy.[[3]](https://en.wikipedia.org/wiki/Southern_blot#cite_note-3)

Method[[edit](https://en.wikipedia.org/w/index.php?title=Southern_blot&action=edit&section=1)]

1. Restriction [endonucleases](https://en.wikipedia.org/wiki/Endonuclease) are used to cut high-molecular-weight DNA strands into smaller fragments.
2. The DNA fragments are then [electrophoresed](https://en.wikipedia.org/wiki/Gel_electrophoresis) on an [agarose gel](https://en.wikipedia.org/wiki/Agarose_gel_electrophoresis) to separate them by size.
3. If some of the DNA fragments are larger than 15 [kb](https://en.wikipedia.org/wiki/Base_pair#Length_measurements), then prior to blotting, the gel may be treated with an acid, such as dilute [HCl](https://en.wikipedia.org/wiki/Hydrochloric_acid). This [depurinates](https://en.wikipedia.org/wiki/Depurination) the DNA fragments, breaking the DNA into smaller pieces, thereby allowing more efficient transfer from the gel to membrane.
4. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (typically containing [sodium hydroxide](https://en.wikipedia.org/wiki/Sodium_hydroxide)) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged thymine residues of DNA to a positively charged amino groups of membrane, separating it into single DNA strands for later [hybridization](https://en.wikipedia.org/wiki/Nucleic_acid_hybridization) to the probe (see below), and destroys any residual RNA that may still be present in the DNA. The choice of alkaline over neutral transfer methods, however, is often empirical and may result in equivalent results.[[*citation needed*](https://en.wikipedia.org/wiki/Wikipedia%3ACitation_needed)]
5. A sheet of [nitrocellulose](https://en.wikipedia.org/wiki/Nitrocellulose) (or, alternatively, [nylon](https://en.wikipedia.org/wiki/Nylon)) [membrane](https://en.wikipedia.org/wiki/Artificial_membrane) is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. If transferring by suction, [20X SSC](https://en.wikipedia.org/wiki/SSC_buffer) buffer is used to ensure a seal and prevent drying of the gel. Buffer transfer by [capillary action](https://en.wikipedia.org/wiki/Capillary_action) from a region of high [water potential](https://en.wikipedia.org/wiki/Water_potential) to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA from the gel onto the membrane; [ion exchange](https://en.wikipedia.org/wiki/Ion_exchange) interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
6. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours (standard conditions; nitrocellulose or nylon membrane) or exposed to [ultraviolet radiation](https://en.wikipedia.org/wiki/Ultraviolet_radiation) (nylon membrane) to permanently attach the transferred DNA to the membrane.
7. The membrane is then exposed to a [hybridization probe](https://en.wikipedia.org/wiki/Hybridization_probe)—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labelled so that it can be detected, usually by incorporating [radioactivity](https://en.wikipedia.org/wiki/Radioactivity) or tagging the molecule with a [fluorescent](https://en.wikipedia.org/wiki/Fluorescence) or [chromogenic dye](https://en.wikipedia.org/wiki/Chromogenic_in_situ_hybridization). In some cases, the hybridization probe may be made from RNA, rather than DNA. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon or herring sperm DNA for blocking of the membrane surface and target DNA, deionized [formamide](https://en.wikipedia.org/wiki/Formamide), and detergents such as [SDS](https://en.wikipedia.org/wiki/Sodium_dodecyl_sulfate) to reduce non-specific binding of the probe.
8. After hybridization, excess probe is washed from the membrane (typically using [SSC buffer](https://en.wikipedia.org/wiki/SSC_buffer)), and the pattern of hybridization is visualized on [X-ray](https://en.wikipedia.org/wiki/X-ray) film by [autoradiography](https://en.wikipedia.org/wiki/Autoradiography) in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.

Result

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. It also allows for the fixation of the target-probe hybrids, required for analysis by [autoradiography](https://en.wikipedia.org/wiki/Autoradiography) or other detection methods. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a [genome](https://en.wikipedia.org/wiki/Genome). A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (for example, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

Applications

Southern blotting transfer may be used for homology-based cloning on the basis of amino acid sequence of the protein product of the target gene. [Oligonucleotides](https://en.wikipedia.org/wiki/Oligonucleotide) are designed so that they are similar to the target sequence. The oligonucleotides are chemically synthesized, radiolabeled, and used to screen a [DNA library](https://en.wikipedia.org/wiki/DNA_library), or other collections of cloned DNA fragments. Sequences that hybridize with the hybridization probe are further analysed, for example, to obtain the full length sequence of the targeted gene.

Southern blotting can also be used to identify methylated sites in particular genes. Particularly useful are the restriction nucleases *MspI* and *HpaII*, both of which recognize and cleave within the same sequence. However, *HpaII* requires that a C within that site be methylated, whereas *MspI* cleaves only DNA unmethylated at that site. Therefore, any methylated sites within a sequence analyzed with a particular probe will be cleaved by the former, but not the latter, enzyme.[[](https://en.wikipedia.org/wiki/Southern_blot#cite_note-4)

**The northern blot**

The **northern blot**, or RNA blot,[[1]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Gilbert2000-1) is a technique used in [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology) research to study [gene expression](https://en.wikipedia.org/wiki/Gene_expression) by detection of [RNA](https://en.wikipedia.org/wiki/RNA) (or isolated [mRNA](https://en.wikipedia.org/wiki/MRNA)) in a sample.[[2]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Alberts2008-2)[[3]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Kevil1997-3)

With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression rates during differentiation and [morphogenesis](https://en.wikipedia.org/wiki/Morphogenesis), as well as in abnormal or diseased conditions.[[4]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Schlamp2008-4) Northern blotting involves the use of [electrophoresis](https://en.wikipedia.org/wiki/Electrophoresis) to separate RNA samples by size, and detection with a [hybridization probe](https://en.wikipedia.org/wiki/Hybridization_probe) complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting.[[5]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Trayhurn1996-5) The northern blot technique was developed in 1977 by James Alwine, [David Kemp](https://en.wikipedia.org/wiki/David_Kemp_%28Australian_scientist%29), and George Stark at [Stanford University](https://en.wikipedia.org/wiki/Stanford_University),[[6]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-6) with contributions from Gerhard Heinrich. Northern blotting takes its name from its similarity to the first blotting technique, the [Southern blot](https://en.wikipedia.org/wiki/Southern_blot), named for biologist [Edwin Southern](https://en.wikipedia.org/wiki/Edwin_Southern).[[2]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Alberts2008-2) The major difference is that RNA, rather than [DNA](https://en.wikipedia.org/wiki/DNA), is analyzed in the northern blot.[[7]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Bor2006-7)

Procedure[[edit](https://en.wikipedia.org/w/index.php?title=Northern_blot&action=edit&section=1)]

A general blotting procedure[[5]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Trayhurn1996-5) starts with extraction of total RNA from a homogenized tissue sample or from cells. Eukaryotic mRNA can then be isolated through the use of oligo (dT) cellulose [chromatography](https://en.wikipedia.org/wiki/Chromatography) to isolate only those RNAs with a [poly(A) tail](https://en.wikipedia.org/wiki/Poly%28A%29_tail).[[8]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Durand1993-8)[[9]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Mori1991-9) RNA samples are then separated by gel electrophoresis. Since the gels are fragile and the probes are unable to enter the matrix, the RNA samples, now separated by size, are transferred to a nylon membrane through a capillary or vacuum blotting system.



Capillary blotting system setup for the transfer of RNA from an electrophoresis gel to a blotting membrane.

A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains [formamide](https://en.wikipedia.org/wiki/Formamide) because it lowers the annealing temperature of the probe-RNA interaction, thus eliminating the need for high temperatures, which could cause RNA degradation.[[10]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Yang1993-10) Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) The membrane is washed to ensure that the probe has bound specifically and to prevent background signals from arising. The hybrid signals are then detected by X-ray film and can be quantified by [densitometry](https://en.wikipedia.org/wiki/Densitometry). To create controls for comparison in a northern blot, samples not displaying the gene product of interest can be used after determination by [microarrays](https://en.wikipedia.org/wiki/Microarrays) or [RT-PCR](https://en.wikipedia.org/wiki/RT-PCR).[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11)

**Gels**[[edit](https://en.wikipedia.org/w/index.php?title=Northern_blot&action=edit&section=2)]



RNA run on a formaldehyde agarose gel to highlight the 28S (top band) and 18S (lower band) ribosomal subunits.

The RNA samples are most commonly separated on [agarose](https://en.wikipedia.org/wiki/Agarose) gels containing [formaldehyde](https://en.wikipedia.org/wiki/Formaldehyde) as a denaturing agent for the RNA to limit secondary structure.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11)[[12]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Yamanka1997-12) The gels can be stained with [ethidium bromide](https://en.wikipedia.org/wiki/Ethidium_bromide) (EtBr) and viewed under UV light to observe the quality and quantity of RNA before blotting.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) [Polyacrylamide](https://en.wikipedia.org/wiki/Polyacrylamide) gel electrophoresis with [urea](https://en.wikipedia.org/wiki/Urea) can also be used in RNA separation but it is most commonly used for fragmented RNA or microRNAs.[[13]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Valoczi2004-13) An RNA ladder is often run alongside the samples on an electrophoresis gel to observe the size of fragments obtained but in total RNA samples the ribosomal subunits can act as size markers.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) Since the large ribosomal subunit is 28S (approximately 5kb) and the small ribosomal subunit is 18S (approximately 2kb) two prominent bands appear on the gel, the larger at close to twice the intensity of the smaller.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11)[[14]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Gortner1996-14)

**Probes**[[edit](https://en.wikipedia.org/w/index.php?title=Northern_blot&action=edit&section=3)]

Probes for northern blotting are composed of nucleic acids with a complementary sequence to all or part of the RNA of interest, they can be DNA, RNA, or oligonucleotides with a minimum of 25 complementary bases to the target sequence.[[5]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Trayhurn1996-5) RNA probes (riboprobes) that are transcribed in vitro are able to withstand more rigorous washing steps preventing some of the background noise.[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) Commonly cDNA is created with labelled primers for the RNA sequence of interest to act as the probe in the northern blot.[[15]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Liang1995-15) The probes must be labelled either with radioactive isotopes (32P) or with [chemiluminescence](https://en.wikipedia.org/wiki/Chemiluminescence) in which [alkaline phosphatase](https://en.wikipedia.org/wiki/Alkaline_phosphatase) or [horseradish peroxidase](https://en.wikipedia.org/wiki/Horseradish_peroxidase) (HRP) break down chemiluminescent substrates producing a detectable emission of light.[[16]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Engler-Blum1993-16) The chemiluminescent labelling can occur in two ways: either the probe is attached to the enzyme, or the probe is labelled with a ligand (e.g. [biotin](https://en.wikipedia.org/wiki/Biotin)) for which the ligand (e.g., [avidin](https://en.wikipedia.org/wiki/Avidin) or [streptavidin](https://en.wikipedia.org/wiki/Streptavidin)) is attached to the enzyme (e.g. HRP).[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) X-ray film can detect both the radioactive and chemiluminescent signals and many researchers prefer the chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels.[[16]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Engler-Blum1993-16) The same membrane can be probed up to five times without a significant loss of the target RNA.[[10]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Yang1993-10)

Applications[[edit](https://en.wikipedia.org/w/index.php?title=Northern_blot&action=edit&section=4)]

Northern blotting allows one to observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment.[[9]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Mori1991-9)[[15]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Liang1995-15)[[17]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Baldwin1999-17) The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue,[[11]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Streit2009-11) as well as the gene expression in the rejection of transplanted organs.[[18]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Utans1994-18) If an upregulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding.[[18]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Utans1994-18) The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs.[[8]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Durand1993-8)[[14]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Gortner1996-14) The variance in size of a gene product can also indicate deletions or errors in transcript processing. By altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.[[2]](https://en.wikipedia.org/wiki/Northern_blot#cite_note-Alberts2008-2)

The **western blot**

The **western blot** (sometimes called the **protein immunoblot**), or **western blotting**, is a widely used [analytical technique](https://en.wikipedia.org/wiki/Analytical_technique) in [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology) and [immunogenetics](https://en.wikipedia.org/wiki/Immunogenetics) to detect specific [proteins](https://en.wikipedia.org/wiki/Proteins) in a sample of tissue homogenate or extract.

In brief, the sample undergoes protein [denaturation](https://en.wikipedia.org/wiki/Denaturation_%28biochemistry%29), followed by [gel electrophoresis](https://en.wikipedia.org/wiki/Gel_electrophoresis_of_proteins). A [synthetic](https://en.wikipedia.org/wiki/Synthetic_antibody) or [animal-derived](https://en.wikipedia.org/wiki/Polyclonal_antibodies) [antibody](https://en.wikipedia.org/wiki/Antibody) (known as the [primary antibody](https://en.wikipedia.org/wiki/Primary_antibody)) is created that recognises and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognises and binds to the primary antibody. The secondary antibody is visualised through various methods such as [staining](https://en.wikipedia.org/wiki/Staining), [immunofluorescence](https://en.wikipedia.org/wiki/Immunofluorescence), and radioactivity, allowing indirect detection of the specific target protein.

Other related techniques include [dot blot](https://en.wikipedia.org/wiki/Dot_blot) analysis, [quantitative dot blot](https://en.wikipedia.org/wiki/Quantitative_dot_blot), [immunohistochemistry](https://en.wikipedia.org/wiki/Immunohistochemistry) and [immunocytochemistry](https://en.wikipedia.org/wiki/Immunocytochemistry), where antibodies are used to detect proteins in tissues and cells by [immunostaining](https://en.wikipedia.org/wiki/Immunostaining), and [enzyme-linked immunosorbent assay](https://en.wikipedia.org/wiki/Enzyme-linked_immunosorbent_assay) (ELISA).

The name *western blot* is a play on the [Southern blot](https://en.wikipedia.org/wiki/Southern_blot), a technique for [DNA](https://en.wikipedia.org/wiki/DNA) detection named after its inventor, English biologist [Edwin Southern](https://en.wikipedia.org/wiki/Edwin_Southern). Similarly, detection of RNA is termed as [northern blot](https://en.wikipedia.org/wiki/Northern_blot).[[1]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Stark1977-1) The term "western blot" was given by W. Neal Burnette in 1981,[[2]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Burnette1981-2) although the method itself originated in 1979 in the laboratory of Harry Towbin at the [Friedrich Miescher Institute](https://en.wikipedia.org/wiki/Friedrich_Miescher_Institute_for_Biomedical_Research) in [Basel](https://en.wikipedia.org/wiki/Basel), [Switzerland](https://en.wikipedia.org/wiki/Switzerland).[[3]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Towbin1979-3) Between 1979 and 2019 "it has been mentioned in the titles, abstracts, and keywords of more than 400,000 [PubMed](https://en.wikipedia.org/wiki/PubMed)-listed publications" and may still be the most used protein-analytical technique.

Applications[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=1)]

The western blot is extensively used in [biochemistry](https://en.wikipedia.org/wiki/Biochemistry) for the qualitative detection of single proteins and protein-modifications (such as [post-translational modifications](https://en.wikipedia.org/wiki/Post-translational_modification)). At least 8-9% of all protein-related publications are estimated to apply western blots.[[4]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Moritz2020-4) It is used as a general method to identify the presence of a specific single protein within a complex mixture of proteins. A semi-quantitative estimation of a protein can be derived from the size and color intensity of a protein band on the blot membrane. In addition, applying a [dilution series](https://en.wikipedia.org/w/index.php?title=Dilution_series&action=edit&redlink=1) of a purified protein of known concentrations can be used to allow a more precise estimate of protein concentration. The western blot is routinely used for verification of [protein production](https://en.wikipedia.org/wiki/Protein_production) after [cloning](https://en.wikipedia.org/wiki/Cloning). It is also used in medical diagnostics, e.g., in the [HIV test](https://en.wikipedia.org/wiki/HIV_test) or [BSE](https://en.wikipedia.org/wiki/Bovine_spongiform_encephalopathy)-Test.

The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human [serum](https://en.wikipedia.org/wiki/Blood_plasma) sample. Proteins from known [HIV](https://en.wikipedia.org/wiki/HIV)-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.[[5]](https://en.wikipedia.org/wiki/Western_blot#cite_note-5) A western blot is also used as the definitive test for [variant Creutzfeldt-Jakob Disease](https://en.wikipedia.org/wiki/Variant_Creutzfeldt-Jakob_Disease), a type of prion disease linked to the consumption of contaminated beef from cattle with [Bovine spongiform encephalopathy](https://en.wikipedia.org/wiki/Bovine_spongiform_encephalopathy) (BSE, commonly referred to as 'mad cow disease').[[6]](https://en.wikipedia.org/wiki/Western_blot#cite_note-6)

Another application is in the diagnosis of tularemia. An evaluation of the western blot's ability to detect antibodies against F. tularensis revealed that its sensitivity is almost 100% and the specificity is 99.6%.[[7]](https://en.wikipedia.org/wiki/Western_blot#cite_note-7)

Some forms of [Lyme disease](https://en.wikipedia.org/wiki/Lyme_disease) testing employ western blotting.[[8]](https://en.wikipedia.org/wiki/Western_blot#cite_note-8) A western blot can also be used as a confirmatory test for Hepatitis B infection and HSV-2 (Herpes Type 2) infection.[[9]](https://en.wikipedia.org/wiki/Western_blot#cite_note-9)[[10]](https://en.wikipedia.org/wiki/Western_blot#cite_note-10) In veterinary medicine, a western blot is sometimes used to confirm [FIV](https://en.wikipedia.org/wiki/FIV)+ status in cats.[[11]](https://en.wikipedia.org/wiki/Western_blot#cite_note-11)

Further applications of the western blot technique include its use by the World Anti-Doping Agency [(WADA)](https://en.wikipedia.org/wiki/World_Anti-Doping_Agency). [Blood doping](https://en.wikipedia.org/wiki/Blood_doping) is the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more oxygen to muscles and therefore increase stamina and performance. There are three widely known substances or methods used for blood doping, namely, erythropoietin (EPO), synthetic oxygen carriers and blood transfusions. Each is prohibited under WADA's List of Prohibited Substances and Methods. The western blot technique was used during the 2014 FIFA World Cup in the anti-doping campaign for that event.[[12]](https://en.wikipedia.org/wiki/Western_blot#cite_note-BaumeJan2015-12) In total, over 1000 samples were collected and analyzed by Reichel, et al.[[13]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Reichel2016-13) in the WADA accredited Laboratory of Lausanne, Switzerland. Recent research utilizing the western blot technique showed an improved detection of EPO in blood and urine based on novel Velum SAR precast horizontal gels optimized for routine analysis.[[14]](https://en.wikipedia.org/wiki/Western_blot#cite_note-14) With the adoption of the horizontal SAR-PAGE in combination with the precast film-supported Velum SAR gels the discriminatory capacity of micro-dose application of rEPO was significantly enhanced.

Procedure[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=2)]

The western blot method is composed of a [gel electrophoresis](https://en.wikipedia.org/wiki/Gel_electrophoresis) to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide, followed by an electrophoretic transfer onto a membrane (mostly [PVDF](https://en.wikipedia.org/wiki/PVDF) or [Nitrocellulose](https://en.wikipedia.org/wiki/Nitrocellulose)) and an immunostaining procedure to visualize a certain protein on the blot membrane. [SDS-PAGE](https://en.wikipedia.org/wiki/SDS-PAGE) is generally used for the denaturing electrophoretic separation of proteins. SDS is generally used as a buffer (as well as in the gel) in order to give all proteins present a uniform negative charge, since proteins can be positively, negatively, or neutrally charged. This type of electrophoresis is known as SDS-PAGE (SDS-polyacrylamide gel electrophoresis). Prior to electrophoresis, protein samples are often boiled to denature the proteins present. This ensures that proteins are separated based on size and prevents proteases (enzymes that break down proteins) from degrading samples. Following electrophoretic separation, the proteins are transferred to a membrane (typically [nitrocellulose](https://en.wikipedia.org/wiki/Nitrocellulose) or [PVDF](https://en.wikipedia.org/wiki/PVDF)), where they are blocked with milk (or other blocking agents) to prevent non-specific antibody binding, and then stained with [antibodies](https://en.wikipedia.org/wiki/Antibody) specific to the target protein.[[3]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Towbin1979-3)[[15]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Renart1979-15) Lastly, the membrane will be stained with a secondary antibody that recognizes the first antibody staining, which can then be used for detection by a variety of methods. The gel electrophoresis step is included in western blot analysis to resolve the issue of the [cross-reactivity](https://en.wikipedia.org/wiki/Cross-reactivity) of antibodies.

**Gel electrophoresis**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=3)]

*Main article:*[*Gel electrophoresis*](https://en.wikipedia.org/wiki/Gel_electrophoresis)

The proteins of the sample are separated using [gel electrophoresis](https://en.wikipedia.org/wiki/Gel_electrophoresis). Separation of proteins may be by [isoelectric point](https://en.wikipedia.org/wiki/Isoelectric_point) (pI), [molecular weight](https://en.wikipedia.org/wiki/Molecular_weight), electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel.

By far the most common type of gel electrophoresis employs [polyacrylamide](https://en.wikipedia.org/wiki/Polyacrylamide) gels and buffers loaded with [sodium dodecyl sulfate](https://en.wikipedia.org/wiki/Sodium_dodecyl_sulfate) (SDS). [SDS-PAGE](https://en.wikipedia.org/wiki/SDS-PAGE) (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular mass. Sampled proteins become covered in the negatively charged SDS, effectively becoming anionic, and migrate towards the positively charged (higher voltage) anode (usually having a red wire) through the [acrylamide](https://en.wikipedia.org/wiki/Acrylamide) mesh of the gel. Smaller proteins migrate faster through this mesh, and the proteins are thus separated according to size (usually measured in kilodaltons, [kDa](https://en.wikipedia.org/wiki/KDa)). The concentration of acrylamide determines the resolution of the gel – the greater the acrylamide concentration, the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration, the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into *wells* in the gel. One lane is usually reserved for a *marker* or *ladder*, which is a commercially available mixture of proteins of known molecular weights, typically stained so as to form visible, coloured bands. When [voltage](https://en.wikipedia.org/wiki/Voltage) is applied along the gel, proteins migrate through it at different speeds dependent on their size. These different rates of advancement (different [*electrophoretic mobilities*](https://en.wikipedia.org/wiki/Electrophoretic_mobility)) separate into *bands* within each *lane*. Protein bands can then be compared to the ladder bands, allowing estimation of the protein's molecular weight.



SDS-PAGE electrophoresis

It is also possible to use a [two-dimensional gel](https://en.wikipedia.org/wiki/Two-dimensional_gel_electrophoresis) which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have a neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

**Transfer**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=4)]

To make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of [*nitrocellulose*](https://en.wikipedia.org/wiki/Nitrocellulose)*(NC) or*[*polyvinylidene difluoride*](https://en.wikipedia.org/wiki/Polyvinylidene_difluoride)*(PVDF*). The most commonly used method for transferring the proteins is called [electroblotting](https://en.wikipedia.org/wiki/Electroblotting). Electroblotting uses an electric current to pull the negatively charged proteins from the gel towards the positively charged anode, and into the PVDF or NC membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. An older method of transfer involves placing a membrane on top of the gel, and a stack of filter papers on top of that. The entire stack is placed in a buffer solution which moves up the paper by [capillary action](https://en.wikipedia.org/wiki/Capillary_action), bringing the proteins with it. In practice this method is not commonly used due to the lengthy procedure time.

As a result of either transfer process, the proteins are exposed on a thin membrane layer for detection. Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and cannot withstand repeated probings.



Western blot transfer

**Total protein staining**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=5)]

Total protein staining allows the total protein that has been successfully transferred to the membrane to be visualised, allowing the user to check the uniformity of protein transfer and to perform subsequent normalization of the target protein with the actual protein amount per lane. Normalization with the so-called "loading control" was based on immunostaining of housekeeping proteins in the classical procedure, but is heading toward total protein staining recently, due to multiple benefits.[[16]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Moritz-2017-16) At least seven different approaches for total protein staining have been described for western blot normalization: [Ponceau S](https://en.wikipedia.org/wiki/Ponceau_S), stain-free techniques, Sypro Ruby, [Epicocconone](https://en.wikipedia.org/wiki/Epicocconone), [Coomassie R-350](https://en.wikipedia.org/wiki/Coomassie_Brilliant_Blue), [Amido Black](https://en.wikipedia.org/wiki/Amido_black_10B), and [Cy5](https://en.wikipedia.org/wiki/Cyanine).[[16]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Moritz-2017-16) In order to avoid noise of signal, total protein staining should be performed before blocking of the membrane. Nevertheless, post-antibody stainings have been described as well.[[17]](https://en.wikipedia.org/wiki/Western_blot#cite_note-17)

**Blocking**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=6)]

Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, steps must be taken to prevent the interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein – typically 3–5% [bovine serum albumin](https://en.wikipedia.org/wiki/Bovine_serum_albumin) (BSA) or [non-fat dry milk](https://en.wikipedia.org/wiki/Powdered_milk) (both are inexpensive) in [tris-buffered saline](https://en.wikipedia.org/wiki/Tris-buffered_saline) (TBS) or I-Block, with a minute percentage (0.1%) of detergent such as [Tween 20](https://en.wikipedia.org/wiki/Tween_20) or [Triton X-100](https://en.wikipedia.org/wiki/Triton_X-100). Although non-fat dry milk is preferred due to its availability, an appropriate blocking solution is needed as not all proteins in milk are compatible with all the detection bands.[[18]](https://en.wikipedia.org/wiki/Western_blot#cite_note-:0-18) The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, it cannot bind to the membrane, and therefore the only available binding site is the specific target protein. This reduces background in the final product of the western blot, leading to clearer results, and eliminates false positives.

**Incubation**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=7)]

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate, this enzyme drives a colorimetric reaction and produces a color. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

**Primary antibody**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=8)]

The [primary antibodies](https://en.wikipedia.org/wiki/Primary_antibody) are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a solution of primary antibody (generally between 0.5 and 5 micrograms/mL) diluted in either PBS or TBST wash buffer is incubated with the membrane under gentle agitation for typically an hour at room temperature, or overnight at 4**°**C. The antibody solution is incubated with the membrane for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with lesser temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise"). Following incubation, the membrane is washed several times in wash buffer to remove unbound primary antibody, and thereby minimize background.[[18]](https://en.wikipedia.org/wiki/Western_blot#cite_note-:0-18) Typically, the wash buffer solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA.

**Secondary antibody**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=9)]

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody known as the [secondary antibody](https://en.wikipedia.org/wiki/Primary_and_secondary_antibodies). Antibodies come from animal sources (or animal sourced [hybridoma](https://en.wikipedia.org/wiki/Hybridoma) cultures). The secondary antibody recognises and binds to the species-specific portion of the primary antibody. Therefore, an anti-mouse secondary antibody will bind to almost any mouse-sourced primary antibody, and can be referred to as an 'anti-species' antibody (e.g. anti-mouse, anti-goat etc.). To allow detection of the target protein, the secondary antibody is commonly linked to [biotin](https://en.wikipedia.org/wiki/Biotin) or a reporter [enzyme](https://en.wikipedia.org/wiki/Enzyme) such as [alkaline phosphatase](https://en.wikipedia.org/wiki/Alkaline_phosphatase) or [horseradish peroxidase](https://en.wikipedia.org/wiki/Horseradish_peroxidase). This means that several secondary antibodies will bind to one primary antibody and enhance the signal, allowing the detection of proteins of a much lower concentration than would be visible by SDS-PAGE alone.

[Horseradish peroxidase](https://en.wikipedia.org/wiki/Horseradish_peroxidase) (HRP) is commonly linked to secondary antibodies to allow the detection of the target protein by [chemiluminescence](https://en.wikipedia.org/wiki/Chemiluminescence). The chemiluminscent substrate is cleaved by HRP, resulting in the production of [luminescence](https://en.wikipedia.org/wiki/Luminescence). Therefore, the production of luminescence is proportional to the amount of HRP-conjugated secondary antibody, and therefore, indirectly measures the presence of the target protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a [4-chloronaphthol](https://en.wikipedia.org/w/index.php?title=4-chloronaphthol&action=edit&redlink=1) stain with 1% [hydrogen peroxide](https://en.wikipedia.org/wiki/Hydrogen_peroxide); the reaction of peroxide radicals with 4-chloronaphthol produces a dark purple stain that can be photographed without using specialized photographic film.



Western blot binding

As with the [ELISPOT](https://en.wikipedia.org/wiki/ELISPOT) and [ELISA](https://en.wikipedia.org/wiki/ELISA) procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane (see the figure below with blue bands).

Another method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. The light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in the signal produced by labeled antibodies bound to proteins on a western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state.[[19]](https://en.wikipedia.org/wiki/Western_blot#cite_note-Ambroz2006-19)

A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like [*Staphylococcus*](https://en.wikipedia.org/wiki/Staphylococcus) Protein A or Streptavidin with a radioactive isotope of iodine. Since other methods are safer, quicker, and cheaper, this method is now rarely used; however, an advantage of this approach is the sensitivity of auto-radiography-based imaging, which enables highly accurate protein quantification when combined with optical software (e.g. Optiquant).

**One step**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=10)]

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, reduction of cost, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with fewer consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known [protein tags](https://en.wikipedia.org/wiki/Protein_tags). The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.



Western blot using radioactive detection system

**Detection and visualization**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=11)]

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is commonly repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is [normalized](https://en.wikipedia.org/wiki/Normalization_%28statistics%29) to the structural protein to control between groups. A superior strategy is the normalization to the total protein visualized with trichloroethanol[[20]](https://en.wikipedia.org/wiki/Western_blot#cite_note-20)[[21]](https://en.wikipedia.org/wiki/Western_blot#cite_note-21) or [epicocconone](https://en.wikipedia.org/wiki/Epicocconone).[[22]](https://en.wikipedia.org/wiki/Western_blot#cite_note-22) This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers. (see [western blot normalization](https://en.wikipedia.org/wiki/Western_blot_normalization))

**Colorimetric detection**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=12)]

The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as [peroxidase](https://en.wikipedia.org/wiki/Peroxidase)) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through [densitometry](https://en.wikipedia.org/wiki/Densitometry) (how intense the stain is) or [spectrophotometry](https://en.wikipedia.org/wiki/Spectrophotometry).

**Chemiluminescent detection**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=13)]

[Chemiluminescent](https://en.wikipedia.org/wiki/Chemiluminescence) detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by CCD cameras which capture a digital image of the western blot or photographic film. The use of film for western blot detection is slowly disappearing because of non linearity of the image (non accurate quantification). The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.



Western blot chemiluminescent detection

**Radioactive detection**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=14)]

Radioactive labels do not require enzyme substrates, but rather, allow the placement of medical X-ray film directly against the western blot, which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image above). The importance of radioactive detections methods is declining due to its hazardous radiation[[*citation needed*](https://en.wikipedia.org/wiki/Wikipedia%3ACitation_needed)], because it is very expensive, health and safety risks are high, and ECL (enhanced chemiluminescence) provides a useful alternative.

**Fluorescent detection**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=15)]

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as a CCD camera equipped with appropriate emission filters which captures a digital image of the western blot and allows further data analysis such as molecular weight analysis and a quantitative western blot analysis. Fluorescence is considered to be one of the best methods for quantification but is less sensitive than chemiluminescence.[[23]](https://en.wikipedia.org/wiki/Western_blot#cite_note-23)

**Secondary probing**[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=16)]

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

2-D gel electrophoresis[[edit](https://en.wikipedia.org/w/index.php?title=Western_blot&action=edit&section=17)]

*Main article:*[*Two-dimensional gel electrophoresis*](https://en.wikipedia.org/wiki/Two-dimensional_gel_electrophoresis)

Two-dimensional SDS-PAGE uses the principles and techniques outlined above. 2-D SDS-PAGE, as the name suggests, involves the migration of polypeptides in 2 dimensions. For example, in the first dimension, polypeptides are separated according to [isoelectric point](https://en.wikipedia.org/wiki/Isoelectric_point), while in the second dimension, polypeptides are separated according to their [molecular weight](https://en.wikipedia.org/wiki/Molecular_weight). The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine, arginine) and negatively (e.g. glutamate, aspartate) charged amino acids, with negatively charged amino acids contributing to a low isoelectric point and positively charged amino acids contributing to a high isoelectric point. Samples could also be separated first under nonreducing conditions using SDS-PAGE, and under reducing conditions in the second dimension, which breaks apart disulfide bonds that hold subunits together. SDS-PAGE might also be coupled with urea-PAGE for a 2-dimensional gel.

In principle, this method allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different [isoforms](https://en.wikipedia.org/wiki/Isoforms) of a particular protein – e.g. a protein that has been phosphorylated (by addition of a negatively charged group). Proteins that have been separated can be cut out of the gel and then analysed by [mass spectrometry](https://en.wikipedia.org/wiki/Mass_spectrometry), which identifies their molecular weigh