

A REVIEW ON IMMUNOBLOTTING TECHNIQUES

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Article Received on: 12/06/2025

Article Revised on: 01/07/2025

Article Accepted on: 22/07/2025



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ABSTRACT

Blotting techniques such as Western, Southern, and Northern blotting are essential molecular biology tools used for detecting specific proteins, DNA, and RNA, respectively. Among them, immunoblotting (Western blotting) is widely utilized for protein analysis due to its high specificity, combining electrophoresis, membrane transfer, and antibody-based detection. This review outlines the principles, procedures, and applications of each blotting method, highlighting their roles in diagnostics, gene expression studies, and biomarker discovery. Additionally, the limitations of immunoblotting are discussed alongside advancements aimed at improving sensitivity, throughput, and reproducibility. These techniques continue to evolve, maintaining their significance in both research and clinical settings.

KEYWORDS: Immunoblotting, Western blotting, Blotting techniques, Protein detection, Southern blotting, Northern blotting, Molecular diagnostics, Antibody-based detection, Gene expression, Biomedical research.

Introduction to Immunoblotting Technique

Immunoblotting, commonly referred to as Western blotting, is a powerful and widely employed technique for the detection and analysis of specific proteins within complex biological samples. This method combines gel electrophoresis, membrane transfer, and antibody-based detection, allowing for high specificity and accuracy in protein identification. Since its development in the late 1970s by Towbin et al., immunoblotting has become an indispensable tool in biomedical research, clinical diagnostics, and proteomics.

The standard immunoblotting workflow consists of three essential steps.

Protein Separation – Proteins are separated based on their molecular weight using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis).

Membrane Transfer – The resolved proteins are transferred from the gel onto a nitrocellulose or PVDF membrane, preserving their structure for subsequent detection.

Antibody-Based Detection – The target protein is identified using a primary antibody specific to it, followed by a secondary antibody conjugated with an enzyme or fluorophore for signal amplification. Detection is achieved via chemiluminescence, fluorescence, or colorimetric methods.

Western blotting has been pivotal in disease diagnostics, particularly in identifying biomarkers for cancer, neurodegenerative diseases, and infectious diseases such as HIV. With continued advancements in high-resolution imaging, fluorescent multiplexing, and mass spectrometry integration, immunoblotting now offers improved sensitivity, specificity, and semi-quantitative analysis, solidifying its role as a cornerstone in molecular and clinical biology.^[3,4]

Overview of Blotting Techniques

Blotting is a fundamental molecular biology technique used to detect specific biomolecules—DNA, RNA, or proteins—by separating them via electrophoresis and transferring them onto a solid membrane for analysis. This approach allows for targeted identification, visualization, and characterization of biological molecules and has been crucial in both basic research and clinical applications.

There are three classical types of blotting techniques.

Southern Blotting – Developed by Edwin Southern in 1975, used for detecting specific DNA sequences.

Northern Blotting – Introduced by Alwine et al. in 1977, used for RNA detection and quantification.

Western Blotting – Described by Towbin et al. in 1979, used for the detection of proteins through antibody-based binding.^[2,8]

Each blotting technique involves common steps.

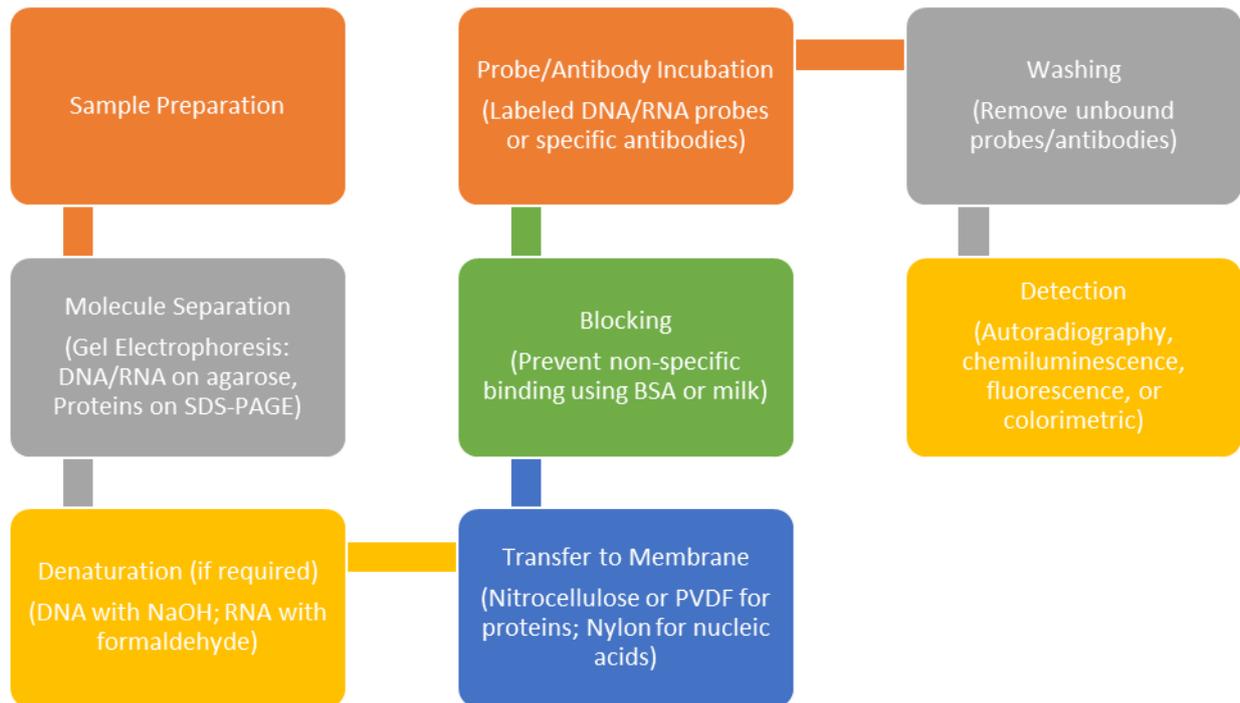


Figure 01: Common steps of blotting technique.

Types of Immunoblotting Techniques^[14]

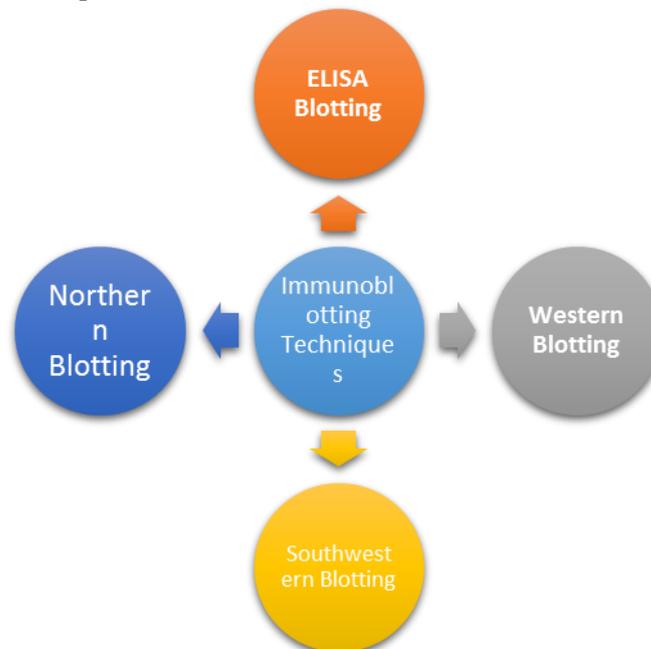


Figure 02: Types of Immunoblotting Techniques.

Enzyme-linked immunosorbent assay (ELISA)

History of ELISA

ELISA (Enzyme-Linked Immunosorbent Assay) was developed in 1971 by Peter Perlmann and Eva Engvall as a safer alternative to radioimmunoassay (RIA), which used radioactive labels. Instead, ELISA uses enzymes

like alkaline phosphatase or horseradish peroxidase to produce a color change for antigen detection. Since then, it has evolved into various formats (e.g., sandwich, competitive, indirect ELISAs) and has been widely used in disease diagnosis, immunology research, and pharmaceutical development.

Principle of ELISA

The enzyme-linked immunosorbent assay (ELISA) is a sensitive, high-throughput method used to detect and quantify specific antigens in liquid samples using antibodies. A key feature of ELISA is the immobilization of one component on a solid surface, typically a polystyrene plate, allowing for effective washing of unbound material. Detection is achieved using enzyme- or fluorescence-labeled antibodies, with signal intensity correlating to antigen concentration. ELISA is particular, quantitative, and adaptable to various sample types, making it widely used in both research and diagnostics. This guide outlines ELISA formats, experimental design, key controls, protocols, and troubleshooting tips.

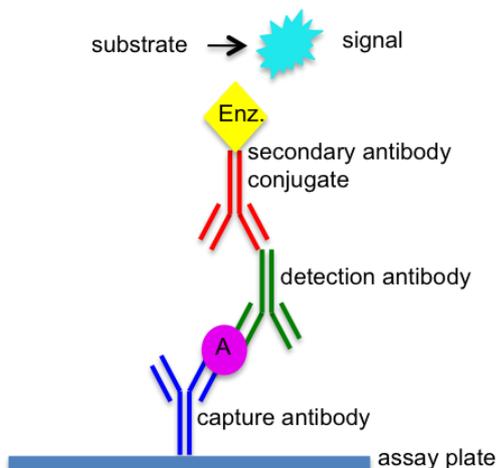


Diagram 01: Enzyme-linked immunosorbent assay (ELISA).

Types of ELISA

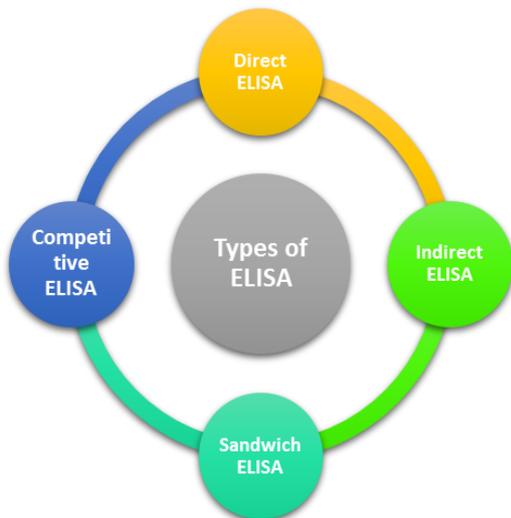
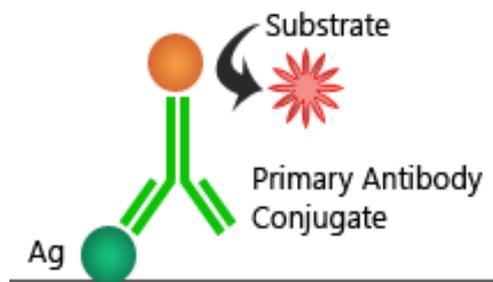


Figure 03: Types of ELISA.

Direct ELISA

In a direct ELISA, the antigen of interest is first immobilized directly onto the surface of a multi-well plate. An antibody that is specific to the antigen and directly conjugated to an enzyme (such as horseradish peroxidase, HRP) is then added. This enzyme reacts with

a substrate to produce a color change, indicating the antigen's presence. This method is fast and involves fewer steps, reducing the chance for error, but it has lower sensitivity because only one labeled antibody binds per antigen, resulting in a weaker signal.^[15]

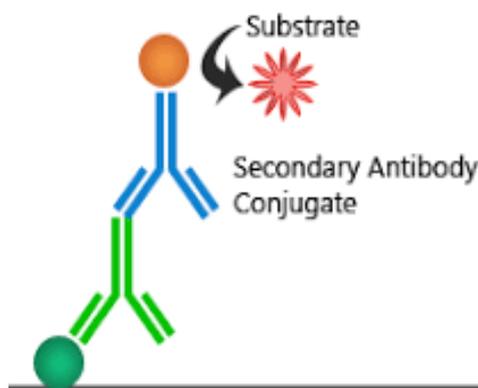


DIRECT ELISA

Diagram 02: Direct ELISA.

Indirect ELISA

Indirect ELISA also begins by coating the antigen onto the plate. A specific, unlabeled primary antibody is added to bind the antigen. After washing, a secondary antibody that is enzyme-linked and specific to the species of the primary antibody is added. This approach amplifies the signal because multiple secondary antibodies can bind to each primary antibody, increasing sensitivity. Indirect ELISA is also more versatile and cost-effective, as one labeled secondary antibody can be used with many different primary antibodies from the same host species.



INDIRECT ELISA

Diagram 03: Indirect ELISA.

Sandwich ELISA

Sandwich ELISA starts by coating the plate with a capture antibody that is specific to the target antigen.

When the sample is added, only the desired antigen binds to this antibody, while other sample components are washed away. Next, a second detection antibody is added, which binds to a different epitope on the same antigen. This detection antibody may be directly enzyme-linked (direct sandwich) or followed by a labeled secondary antibody (indirect sandwich). Sandwich ELISAs offer high specificity and sensitivity, making them ideal for complex samples, but require well-matched antibody pairs that don't interfere with each other's binding.

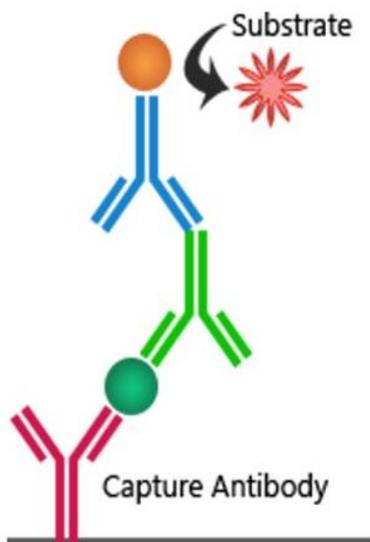


Diagram 04: Sandwich ELISA.

Competitive ELISA

Competitive ELISA, also known as inhibition ELISA, is a sensitive method used when the antigen is small or only has one epitope, making it difficult to use two antibodies as in other ELISA types. Unlike standard ELISA formats, the signal in competitive ELISA decreases as the amount of target antigen increases. This happens because the sample antigen competes with a labeled control antigen for binding to a limited amount of antibody. If more sample antigen is present, fewer labeled control antigen can bind, resulting in a lower signal. This format is useful when matched antibody pairs are not available and works well with crude samples. It requires the use of monoclonal antibodies to ensure accurate detection of a single epitope.

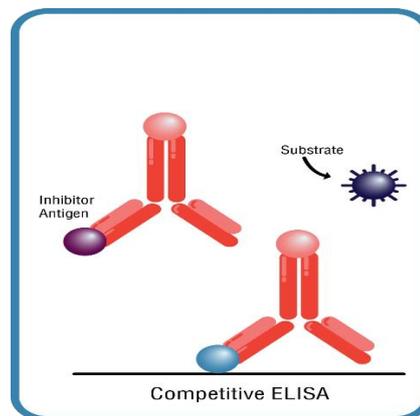


Diagram 05: Competitive ELISA.

Procedure

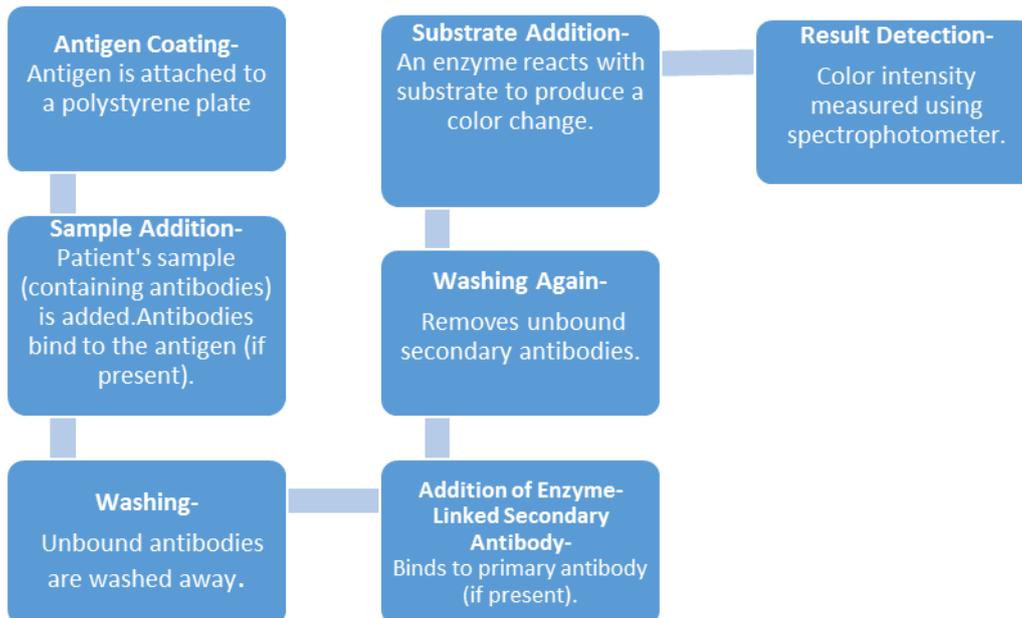


Figure 04: Procedure ELISA.

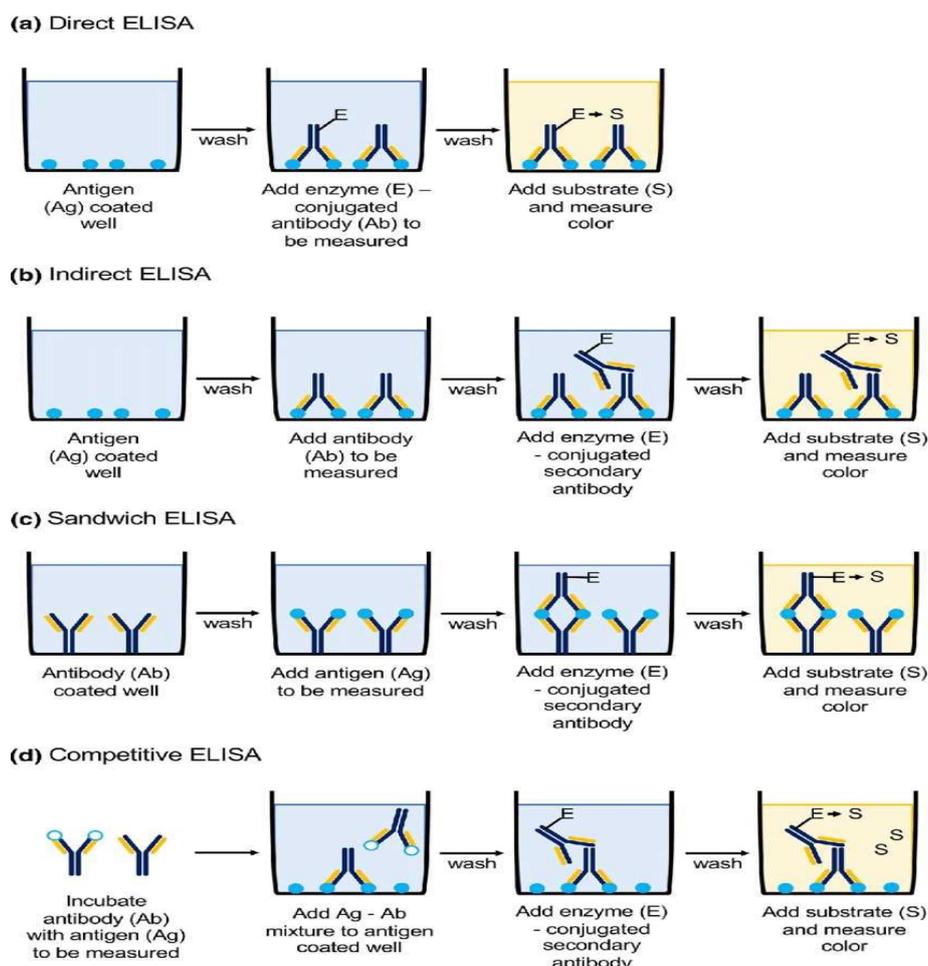


Diagram 06: Process of ELISA.

applications of ELISA

- Serum Antibody Concentrations.
- Detecting potential food allergens. (milk, peanuts, walnuts, almonds and eggs).
- Disease outbreaks- tracking the spread of disease. (e.g., HIV, bird flu, common colds, cholera, STDs, etc).
- Detections of antigens. (e.g., pregnancy hormones, drug allergens, GMO, mad cow disease).
- Detection of antibodies in a blood sample for past exposure to the disease. (e.g., Lyme Disease, trichinosis, HIV, bird flu).^[18]

advantages of ELISA

- Fast-90 samples tested in 2-3 hours.
- Sensitivity (up to 10 pg/ml).
- Specificity (sample with high concentration of contaminants).
- Many samples can be processed at once.
- Small sample size required (10 uL ~ 100 uL).

- Colorimetric results - easily observed and measured (spectrophotometer).
- Test for the presence of Ag or Ab.
- Flexible usage for research design.
- Easy to learn, simple procedure.

1. Western Blotting (Protein Detection)

history of western blotting

Western blotting was developed in 1979 by Harry Towbin et al. and later refined by W. Neal Burnette in 1981, who coined the term. Inspired by Southern blotting (DNA) and Northern blotting (RNA), it became a key technique for protein detection in research and diagnostics, evolving with advancements in imaging and detection methods.

principle of western blotting

Western blotting is a technique specifically used for the detection and analysis of proteins within a sample. This method provides high specificity in identifying proteins based on their molecular weight and antibody binding.

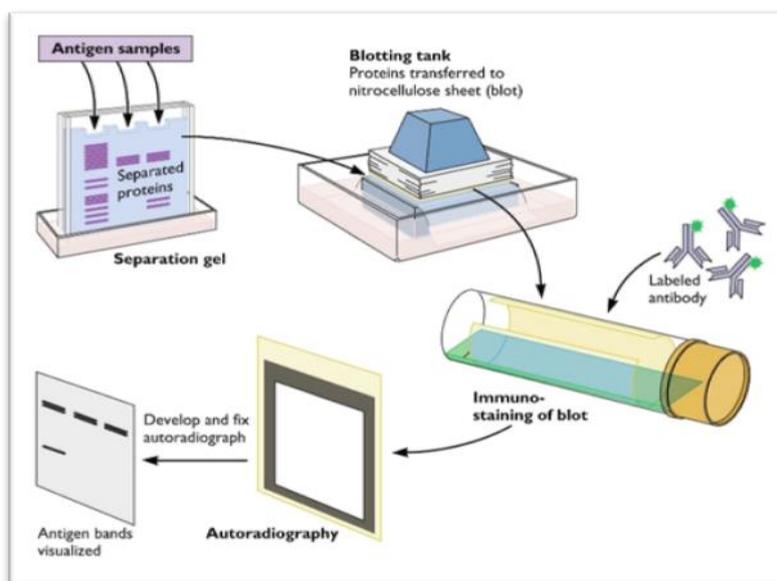


Diagram 07: Western Blotting.

This diagram illustrates the Western blotting technique, a widely used immunoblotting method for detecting

specific proteins in a sample. The process involves the following key steps.

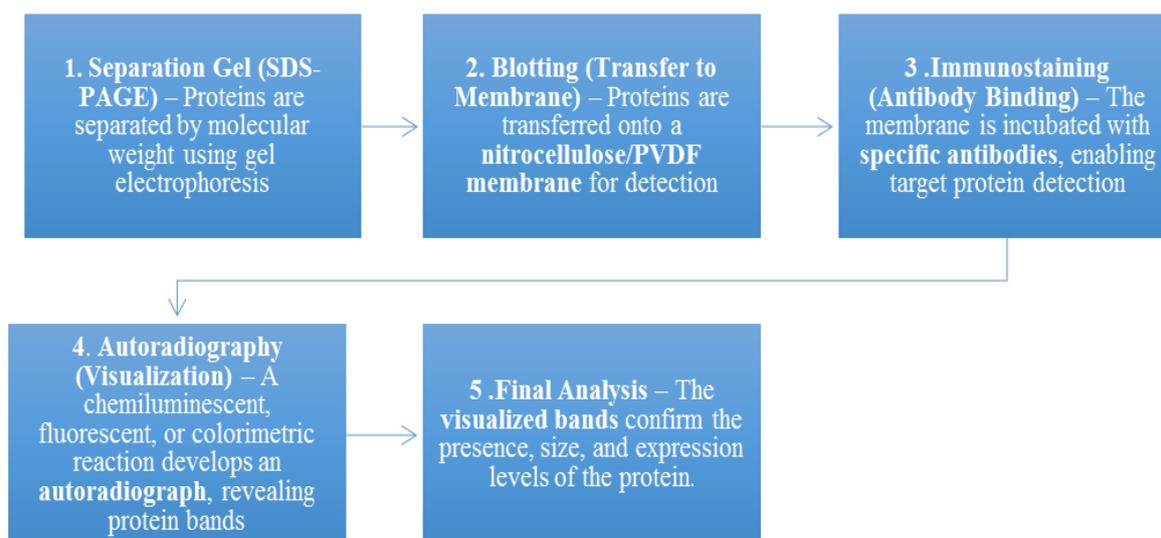


Figure 04: Procedure western blotting.

applications of western blotting

- Disease diagnosis (e.g., HIV, Lyme disease).
- Protein expression studies in research.
- Detection of post-translational modifications.
- Verification of recombinant protein production.
- Analysis of IgG fractions purified from human plasma.
- Confirmatory test for hepatitis-B involves western blotting technique.
- Western blotting technique is used in definitive test for BSE, which is commonly known as mad cow disease.
- This technique as employed in the gene expression studies.^[3,12,17]

limitations of western blotting

- Very delicate and time consuming process.
- If a protein is degraded quickly, then western blotting technique won't detect it well.
- Well trained techniques are required for this technique.
- Primary antibody availability is crucial.

2. Southern Blotting (DNA Detection)

Southern blotting, developed by E.M. Southern in 1975, is used for detecting specific DNA sequences in a complex mixture. It helps in genetic analysis, mutation detection, and forensic studies.

principle of southern blotting

Southern blotting detects specific DNA sequences by digesting DNA with restriction enzymes, separating fragments via gel electrophoresis, and transferring them

onto a membrane. A labeled DNA probe binds to the target sequence, producing a detectable signal for identification.

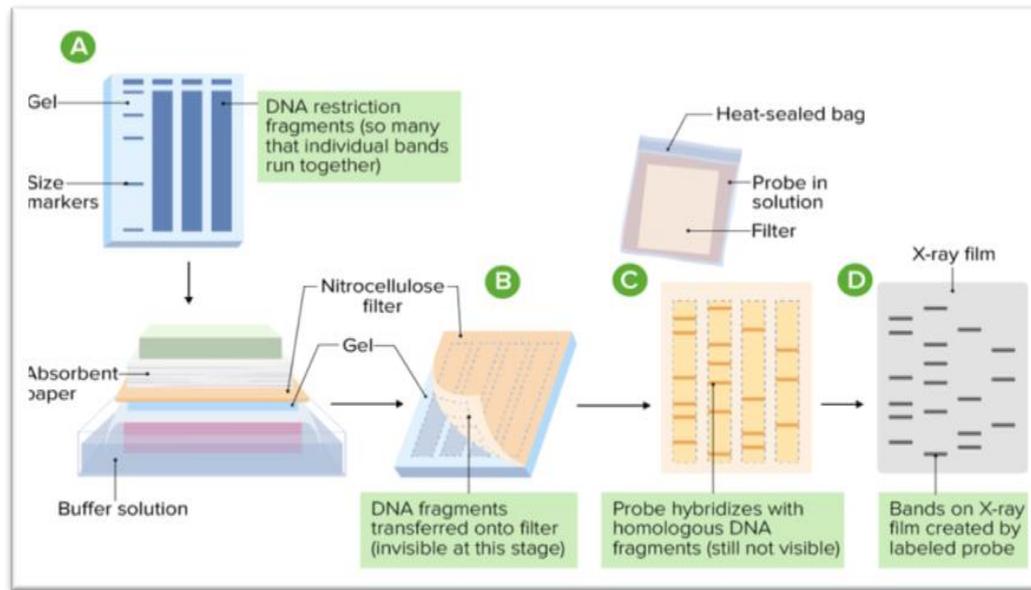


Diagram 08: Southern Blotting.

steps involved in southern blotting

- DNA Extraction – Genomic DNA is isolated from a sample.
- Restriction Digestion – DNA is cut into fragments using restriction enzymes.
- Gel Electrophoresis – DNA fragments are separated based on size using agarose gel electrophoresis.
- Denaturation – DNA is treated with an alkaline solution (NaOH) to convert double-stranded DNA into single strands.
- Transfer to Membrane – The denatured DNA is transferred onto a nylon or nitrocellulose membrane.
- Hybridization with Probe – A radioactively or fluorescently labeled DNA probe complementary to the target sequence is applied.
- Washing & Detection – Excess probes are washed off, and the hybridized DNA is detected using autoradiography or chemiluminescence.

applications of southern blotting

- Genetic disorder detection (e.g., sickle cell anemia, cystic fibrosis).
- DNA fingerprinting in forensic investigations.
- Gene mapping and mutation analysis.
- Identifying specific DNA in a DNA sample.
- Preparation of RFLP (Restriction Fragment Length Polymorphism) maps.
- Detection and identification of trans gene in transgenic individuals.
- For the diagnosis of infectious diseases.
- Determination of the molecular weight of restriction fragments and to measure relative amounts in different samples.^[1,16]

limitations of southern blotting

- Time-consuming and labor-intensive
- Requires large amounts of high-quality DNA
- Limited sensitivity (not ideal for detecting low-abundance sequences)
- Semi-quantitative, not precise for quantification
- Complex and specific probe design needed
- Often involves radioactive materials (safety concerns)
- Requires prior knowledge of the target DNA sequence
- Expensive equipment and reagents
- Not suitable for high-throughput analysis
- Cannot detect novel or unknown DNA sequences

3. Northern Blotting (RNA Detection)**principle of northern blotting**

Northern blotting, introduced by Alwine et al. in 1977, is used for the detection and quantification of RNA molecules. This technique is essential in gene expression studies.

Northern blotting is a hybridization-based method used to detect specific RNA sequences. It involves separating RNA molecules based on their size (molecular weight) through gel electrophoresis and transferring them onto a nylon membrane. Detection occurs using a labeled hybridization probe (radioactive or chemiluminescent) that is complementary to the target mRNA sequence.

steps involved in northern blotting

- RNA Extraction – Total RNA or mRNA is isolated from a biological sample.

- Gel Electrophoresis – RNA molecules are separated based on size using agarose gel electrophoresis with formaldehyde to prevent secondary structure formation.
- Transfer to Membrane – The separated RNA is transferred onto a nylon or nitrocellulose membrane.
- Hybridization with Probe – A complementary labeled RNA or DNA probe binds to the target RNA sequence.
- Washing & Detection – Unbound probes are washed off, and hybridized RNA is visualized using autoradiography or chemiluminescence.

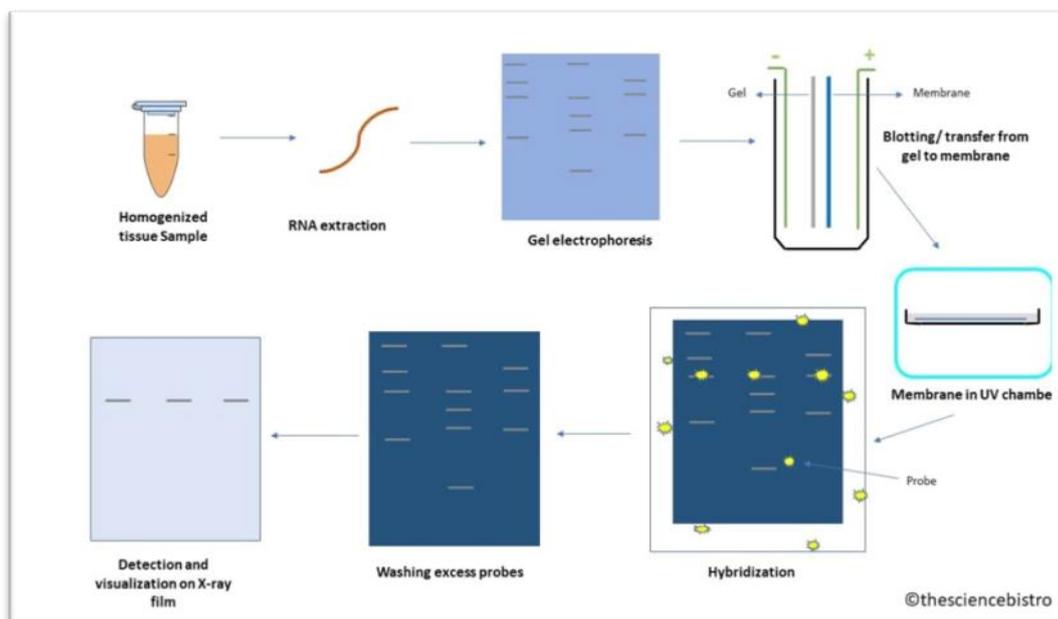


Diagram 09: Northern Blotting.

applications of northern blotting

- Measuring gene expression in different tissues or conditions.
- Detecting viral RNA in infections.
- Studying RNA splicing and modifications.
- Northern blot is a valuable method used by researchers to determine gene expression patterns between tissues, organs, developmental stages, pathogen infections, and throughout treatment.
- The technique has been used to demonstrate the overexpression of oncogenes and the downregulation of tumor-suppressor genes in cancerous cells compared to normal tissue.
- The gene product's size variance can 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.

- Used in the screening of recombinants by detecting the mRNA produced by the transgene.^[9,17]

limitations of northern blotting

- Time-consuming and technically demanding
- Requires large amounts of high-quality RNA
- RNA is easily degraded
- Low sensitivity
- Semi-quantitative
- Poor detection of low-abundance transcripts
- Use of radioactive or hazardous chemicals
- Low throughput
- Expensive equipment and reagents
- Not suitable for small RNAs (e.g., miRNAs) without special protocols

Applications of Immunoblotting.^[4,5,9,13]

Application	Purpose	Example Use Case
Disease Diagnosis	Confirm the presence of disease-specific antigens	HIV, Lyme disease confirmation
Protein Expression Analysis	Measure relative protein abundance under various conditions	Oncogene expression in cancer studies
Post-translational Modification	Detect changes like phosphorylation, glycosylation, or ubiquitination	p53 phosphorylation detection
Epitope Mapping	Identify antibody binding regions on antigens	Vaccine target identification
Subcellular Localization	Determine protein distribution in cellular	Mitochondrial vs. cytoplasmic

	compartments	localization
Antibody Validation	Test specificity and performance of antibodies	Monoclonal antibody screening
Quality Control of Therapeutics	Verify identity and purity of therapeutic proteins	Recombinant insulin quality check
Validation of Gene Editing	Confirm successful gene knockout or knockdown	CRISPR/Cas9 validation
Protein-Protein Interaction Study	Detect proteins in complexes using co-IP and Western blot	MAPK pathway protein complex analysis
Protein Stability & Degradation	Track protein half-life and degradation under stress or time	Proteasome inhibition studies
Developmental Biology	Analyze protein expression across developmental stages	Transcription factors in embryogenesis
Tissue-Specific Expression	Compare protein levels across different tissues	Tissue-specific biomarker detection
Biomarker Discovery	Identify disease- or condition-specific protein markers	Cancer biomarker profiling
Pathogen Detection	Detect viral or bacterial proteins in samples	Hepatitis B surface antigen detection
Drug Response Monitoring	Assess protein changes in response to treatment	Bcl-2 levels after chemotherapy

Limitations of Immunoblotting.^[4,5,12]

Limitation	Description
Low Sensitivity	May not detect low-abundance proteins without enhanced detection systems.
Semi-Quantitative Nature	Standard Western blotting provides relative, not absolute, protein quantification.
Antibody Dependence	Requires high-quality, specific antibodies; cross-reactivity can cause errors.
Time-Consuming	Multi-step process (gel electrophoresis, transfer, blocking, probing, washing) takes several hours to days.
Labor Intensive	Manual handling increases variability and error; lacks full automation.
Protein Denaturation	Proteins are denatured during SDS-PAGE, preventing assessment of native structure or function.
Limited Multiplexing	Difficult to probe multiple proteins on the same blot unless using fluorescent or multiplex systems.
Non-Specific Bands	Non-specific binding of antibodies may lead to background noise and false positives.
Transfer Inefficiency	Incomplete protein transfer from gel to membrane can result in underestimation or loss of proteins.
Normalization Issues	Common loading controls (e.g., β -actin, GAPDH) may vary under experimental conditions, affecting accuracy.
Reproducibility Problems	Variability in reagents, antibody batches, and technique leads to inter-lab inconsistency.
Low Throughput	Typically processes few samples at a time; not ideal for large-scale screening.

CONCLUSION

Detection and analysis of specific proteins. Alongside Southern and Northern blotting, these techniques have advanced our understanding of gene and protein expression, disease mechanisms, and diagnostic applications.

Despite certain limitations such as low sensitivity, reliance on antibody quality, and labor-intensive workflows, ongoing innovations continue to enhance their accuracy, throughput, and usability. Together, these blotting methods continue to play a crucial role in research and clinical diagnostics, with evolving technologies further expanding their potential.

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