

Guide to Western Blotting



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INTRODUCTION

The aim of Western blotting is to identify specific proteins within a complex mixture. The technique is commonly used to analyze cell lysate samples and tissue homogenates, and evolved from Southern blotting during the late 1970s. Southern blotting, invented in 1975 by the British biologist Edwin Southern, was designed to identify particular sequences among a mixture of DNA fragments utilizing radioactive probes for detection. Taking their lead from this, early Western blot experiments also used radioactive detection methods (such as ¹²⁵I-labeled protein A) however the Western blotting process has evolved significantly since its inception and is now much quicker and considerably safer.

The Western blot technique requires samples to be resolved on the basis of size through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), following which they are transferred to, and immobilized on, a membrane prior to antibody-based detection.

SAMPLE PREPARATION

Before running a Western blot it is extremely important to research the target protein thoroughly. Several factors should be taken in to account to increase the chances of successful detection:

- **Expression pattern:** The cellular location of the protein should be identified and a lysis buffer of the appropriate strength should be chosen to release it; a nuclear protein may require stronger lysis conditions than a cytoplasmic protein. Some proteins are only expressed under specific conditions, for example at particular stages of the cell cycle or under conditions of hypoxia; if necessary the cells should be treated to enhance expression of the protein to detectable levels.
- **Predicted versus actual protein size:** Although a protein can be given a theoretical molecular weight based on its primary structure, this does not take in to account any post-translational modifications such as phosphorylation or glycosylation which can cause a significant increase in the size of the band that is detected on a Western blot. [UniProt](#) is a useful database where this information can be found.
- **Control samples:** Appropriate controls are critical to correctly identifying the band of interest on a Western blot. A suitable positive control could be a recombinant protein, an over-expression lysate, or a lysate prepared from a cell line that is known to naturally express high levels of the protein of interest. If a recombinant protein is used as a control, it is important to know whether or not it is tagged since the size of the tag will affect the size of the band that is seen on the Western blot. If one of these positive controls is not readily available it may be possible to stimulate expression of the protein of interest by treating the cells.

To prevent degradation of the sample, protein extraction should always be performed under cold conditions with protease inhibitors and phosphatase inhibitors included in the lysis buffer. Ready-to-use inhibitor cocktails are available from a variety of manufacturers, and can also be made in-house; these typically contain inhibitors such as aprotinin, EDTA, EGTA, leupeptin, pepstatin A, PMSF, sodium fluoride and sodium orthovanadate. Samples should be prepared as efficiently as possible, and freeze-thaw cycles should be avoided.

To ensure that all samples will be equivalently loaded on to the gel the protein concentration of each lysate should be determined; a Bradford assay, bicinchoninic acid (BCA) assay or Lowry assay is usually employed. Once the protein concentration has been established, the samples should be normalized with lysis buffer. Sample loading buffer should then be added to each lysate, and at this point it is important to decide whether the samples should be denatured or native. Although many antibodies recognize a contiguous sequence of amino acids, this domain can often lie within the 3-dimensional structure of the protein; under these circumstances it is appropriate to denature the protein. Some antibodies are instead raised against a native protein and will only recognize the epitope as it exists on the surface of the folded protein structure; under these circumstances it is appropriate to run the Western blot under non-denaturing conditions.

When preparing denatured, reduced samples the sample loading buffer will contain glycerol to increase the density of the samples and allow them to sink down in to the wells of the gel upon loading, a tracking dye such as bromophenol blue to enable sample visualization, 2-mercaptoethanol or dithiothreitol (DTT) to reduce disulfide bonds, and SDS to denature the proteins and provide them with a strong negative charge in proportion to their length. These samples should be heated at 95-100°C for 5 minutes prior to loading. Multi-pass membrane proteins are more susceptible to aggregation at high temperatures, and when working with these it may be preferable to heat the samples at a lower temperature for longer. When preparing native, non-reduced samples the SDS and 2-mercaptoethanol or DTT should be omitted from the sample loading buffer and the samples should not be heated.

Equal volumes should be loaded on to the gel to ensure that the samples run in a straight line and to prevent "smiling".

GEL ELECTROPHORESIS

Although it is possible to hand-pour gels, affordable ready-cast gels are available from a wide variety of manufacturers. Low molecular weight proteins are best resolved on high percentage gels (15-20% polyacrylamide), whereas larger proteins are better resolved on low percentage (4-8%) or gradient gels (usually 4-12%). For optimal sample resolution an appropriate running buffer should be selected. The voltage is also important; too high a voltage can overheat the gel and distort the bands.

MEMBRANE TRANSFER

Following separation of the mixture, the proteins should be transferred to a nitrocellulose or PVDF membrane. Nitrocellulose is widely used and is cheaper than PVDF, however PVDF membranes are resilient to mechanical damage and can therefore be more amenable to being stripped and re-probed. Membranes typically have a pore size of 0.45µm, but when working with smaller proteins (<20kDa) a membrane with a pore size of 0.2µm should be chosen. For the transfer of smaller proteins it may be beneficial to remove SDS from the transfer buffer, increase the methanol concentration and reduce the transfer time. For larger proteins the transfer may benefit from increasing the SDS concentration of the transfer buffer, lowering the methanol concentration, or from performing the transfer step overnight at 4°C.

To confirm that the transfer has been successful the membrane can be stained with Ponceau S. For additional confirmation, the gel can be stained with Coomassie Blue to ensure that no protein has been left behind.

IMMUNOBLOTTING

Once the proteins have been transferred to the membrane they can be detected by immunoblotting. At no stage during this process should the membrane be allowed to dry out.

- **Blocking** is a critical step to Western blotting since it prevents non-specific binding of antibodies to the membrane. Non-fat dried milk is often used as a blocking agent since it is inexpensive and widely available yet milk proteins are not compatible with all Western blots; for example it is inadvisable to use milk with anti-phosphoprotein antibodies since milk contains casein which is itself a phosphoprotein that can interfere with the assay readout. Bovine Serum Albumin (BSA) and commercial blocking agents are also commonly used. The blocking agent is usually diluted in either Phosphate Buffered Saline (PBS) or Tris Buffered Saline (TBS), and a low concentration of Tween-20 detergent (commonly 0.1%) is frequently added. The choice of buffer should be optimized during development of the Western blot assay.
- The recommended starting dilution at which the **primary antibody** should be used is usually detailed on the manufacturer's datasheet. Some antibodies give rise to a measurable signal after a one hour incubation at room temperature, while other antibodies perform more favorably after an overnight incubation at 4°C; optimization of the staining conditions should always be carried out. The buffer in which the primary antibody is diluted should also be optimized. In addition to probing the Western blot for the protein of interest, it is essential that loading controls are included to allow interpretation of the data. Actin, GAPDH and tubulin are commonly used for whole cell lysates, while Lamin B1, HDAC1 and Histone H3 are often used as loading controls for nuclear extracts.
- Following incubation of the membrane with primary antibody, the blot should be **washed** thoroughly with large volumes of a suitable buffer; PBS or TBS, with the addition of Tween-20, are often used. The membrane should be agitated gently at room temperature and several changes of the buffer solution should be performed.
- Once the primary antibody has bound to the target protein, it requires detection. The use of radioactive detection methods has evolved to employ anti-species **secondary antibodies**; these are directed against the primary antibody and are often conjugated to an enzyme or a dye. The downstream processing method is dependent on the chosen readout however for accurate results it is critical that the blot is washed thoroughly to remove any unbound label prior to development. It is now possible to directly conjugate the primary antibody, bypassing altogether the use of secondary reagents for detection, and Expedeon offers a wide range of bioconjugation products enabling quick and easy antibody labeling. The detection process is discussed in more detail below.

METHODS OF PROTEIN DETECTION

The handling and disposal issues associated with the use of radioactivity have led to the development of a variety of Western blotting detection methods. Colorimetric, fluorometric and chemiluminescent detection are all well-established techniques, as is the use of gold nanoparticles.

Colorimetric detection relies on the generation of a colored product that becomes deposited on the Western blot; this is formed following the conversion of a chromogenic blotting substrate by an appropriate enzyme. Chromogenic blotting substrates are commercially available in a variety of formats, and the choice of substrate will be dependent on the enzyme label as well as the desired assay sensitivity. Chromogenic substrates for Western blotting with Horseradish Peroxidase (HRP) include DAB, TMB 4CN, ABTS and OPD, while NBT is a commonly used substrate for Glucose Oxidase. BCIP, PNPP and NBT are regularly used with Alkaline Phosphatase.



Figure 1. Schematic representation of colorimetric Western blot detection. The upper panel demonstrates indirect detection while the lower panel shows direct detection. Indirect versus direct detection is discussed in more detail within this guide; Lightning-Link® facilitates direct detection.

The limited sensitivity of chromogenic substrates can make it difficult to optimize them for detecting proteins of low abundance; although the chromogenic reaction can be allowed to develop for several hours (or even overnight) this allows background signal to develop simultaneously. Chromogenic substrates are however perfect for the detection of abundant proteins since the reaction can be monitored visually and allowed to progress until the color development is adequate before being stopped. No specialized equipment is required for visualization of the colored precipitate, and the signal that is produced is highly stable.

- Cost effective
- Easy to determine the exposure time
- No specialized equipment required for detection
- Signal stability of months - years

Fluorometric detection requires the use of an antibody which has been labeled with a fluorophore. A light source is used to excite the fluorophore, which then produces a transient light emission as it returns to its ground state. The light is emitted at a higher wavelength than that which was used for excitation and is detected with a specialized reader.

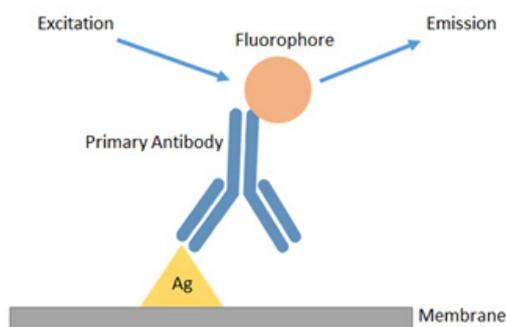


Figure 2. Schematic representation of fluorescent Western blot detection using Lightning-Link®.

Fluorescent reagents are extremely popular for Western blotting and provide the unique advantage of allowing multiplexing to be performed, for example allowing phospho-specific and pan antibodies to be used together on a Western blot despite the protein isoforms sharing very similar molecular weights. This is illustrated in Figure 3.

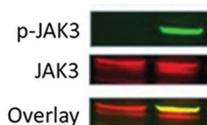


Figure 3. Lysate from untreated (left lane) or IL2-stimulated YT cells (right lane) was resolved by SDS-PAGE and then probed for JAK3 and one of its phosphorylated isoforms. The use of different fluorophores enables the isoforms to be distinguished from one another despite the close similarity in molecular weight.

When multiplexing it is important to choose dyes that do not overlap spectrally in order to avoid background signal. The extinction coefficient and Stokes shift data should also be taken in to account. The extinction coefficient defines how well a fluorophore absorbs light at a particular wavelength; fluorophores with high extinction coefficients are generally brighter. The Stokes shift is the difference between the maximum absorbance and emission wavelengths of a fluorophore; a greater Stokes shift is indicative of less overlap between the two wavelengths.

Unlike colorimetric detection, fluorescent Western blotting can be considered to be quantitative since the signal that is produced is proportional to the amount of target protein which is present. Fluorescent detection is highly sensitive, and since the fluorescent signal is very stable the Western blot can be rescanned several months after it was first generated should this be necessary (provided that the blot has been stored in the dark and not been allowed to dry out).

- Multiplexing is possible - saves time, money and precious sample
- High sensitivity
- Quantitative
- Wide range of fluorophores available
- Signal stability of months - years

Chemiluminescence occurs when a substrate is catalyzed by an enzyme and produces light as a by-product of the reaction. The limiting reagent in the reaction is the substrate; as this is exhausted the light production decreases and eventually stops however a well-optimized procedure should produce a stable light output for several hours allowing consistent and sensitive protein detection.

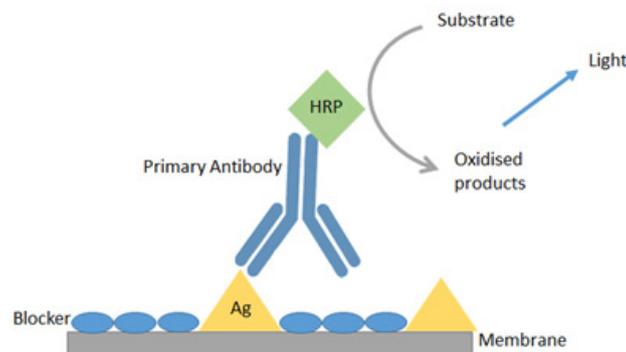


Figure 4. Schematic representation of chemiluminescent Western blot detection using Lightning-Link®.

Chemiluminescent substrates yield the greatest sensitivity of any available Western blotting detection method and their large linear range allows both high and low abundance proteins to be detected and quantified. A wide range of chemiluminescent substrates is commercially available.

- High sensitivity
- Large linear range
- Signal stability of several hours

Gold nanoparticles can be conjugated to antibodies and used for antigen detection. One of the benefits of using gold nanoparticles for Western blot detection is that no downstream processing is required for a signal to be seen by the naked eye. Binding of the gold-conjugated antibody to its immobilized protein target gives rise to a red color on the membrane.

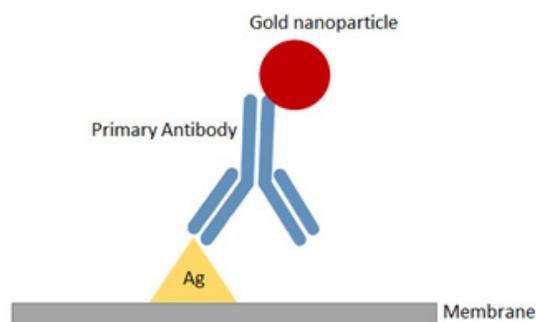


Figure 5. Schematic representation of gold nanoparticle Western blot detection using Lightning-Link®.

The sensitivity of the method can be increased through silver enhancement, during which silver is deposited on the surface of the gold nanoparticles to increase their size and boost the signal.

- No downstream processing necessary
- No specialized equipment required for detection
- Signal stability of months – years following silver enhancement

A wide range of imaging systems is available for measuring Western blotting readouts, and many instruments can be used for multiple forms of detection.

USE OF DIRECTLY-LABELED PRIMARY ANTIBODIES FOR WESTERN BLOTTING

The majority of Western blotting detection methods were developed using secondary antibodies conjugated to the label of interest yet immunoassays can be greatly simplified by directly conjugating the primary antibody to the relevant label, bypassing altogether the need to use secondary antibodies.

The use of a conjugated primary antibody for detection is referred to as direct Western blotting, while the use of a conjugated secondary antibody for detection is known as indirect Western blotting. The popularity of direct Western blotting is steadily increasing, and the advantages of this method are as follows:

- Non-specific binding is avoided since secondary antibodies are not used
- Multiplexing is possible with antibodies from the same species
- Faster since there is no secondary antibody incubation step and therefore fewer wash steps
- Data quality is improved through assay simplification

Lightning-Link® from Expedeon is an innovative technology that enables direct labeling of antibodies, proteins, peptides or any other biomolecule with free amine groups for use in a multitude of applications including Western blotting. The product range includes kits for labeling antibodies with enzymes or with a wide range of fluorophores.

The benefits of Lightning-Link® include:

- Quick and easy to use
- Requires only 30 seconds hands-on time
- No separation steps involved so 100% of the antibody or protein is recovered
- Possibility to label from as little as 10ug to a gram or more of antibody

Despite its apparent simplicity, the Lightning-Link® process is sophisticated and generates conjugates with performance characteristics identical to, or better than, those of conjugates prepared with laborious multistep conjugation procedures.

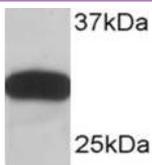
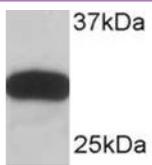
	DIRECTLY LABELED ANTIBODY	INDIRECTLY LABELED ANTIBODY
primary antibody	Goat Anti-NQO1	Goat Anti-NQO1
target	quinone reductase 1	quinone reductase 1
sample lysate	human kidney	human kidney
primary antibody working concentration	0.00425 µg/ml	0.1 µg/ml
secondary antibody used	no (direct conjugation)	yes
exposure time (min)	10	10
primary antibody source	Everest Biotech, Cat no: EB05370	
western blot analysis		

Table 1. Western blot data comparing direct and indirectly-labeled antibodies.

Expedeon also offers the **InnovaCoat® GOLD** product range - one-step gold nanoparticle conjugation kits for antibodies, proteins, peptides or any other biomolecule with an amine group.

The benefits of InnovaCoat® GOLD are as follows:

- Quick and easy to use - covalently link antibodies to gold nanoparticles in just 15 minutes
- Ultra-stable
- Metal-antibody interactions are prevented
- No pH titrations are involved
- Less antibody required than for passive conjugation to gold nanoparticles
- Different surface chemistries available

By eliminating the need for labeled secondary antibodies, Lightning-Link® and InnovaCoat® GOLD provide the potential to create a library of research tools comprising an almost infinite number of conjugates.

For further information on our full range of Lightning-Link® and InnovaCoat® GOLD products, please see our [website](#). View our Western blotting webinar [here](#).

MORE WESTERN BLOTTING PRODUCTS FROM EXPEDEON

LUMIBLUE ECL SOLUTIONS

Enhanced Chemiluminescence (ECL) is a method which provides highly precise detection of proteins labeled either directly or non-directly with Horseradish Peroxidase from Western blots.

- **Linear Response** – Stable signal over range of protein concentrations
- **Stable solution** – Working solutions usable for up to 5 days (LumiBlue ECL Express)
- **Long lasting signal** – Perform multiple exposures with less signal loss
- **Use less Antibody** – High sensitivity results with lower antibody concentration
- **Low Background** – Lower backgrounds regardless of detection method
- **Wide range** – ECL solution suitable for most protein and antibody concentrations
- **Bright Bands** – Better brightness compared to other advanced ECL solutions
- **Standard protocols** – Direct replacement of other ECL solutions with no workflow changes
- **Sensitive detection** – LumiBlue ECL Extreme can detect protein levels as low as 50fg per band
- **Long shelf life** – One year shelf life at 4°C

Our **LumiBlue ECL solutions** product range use unique patented downstream catalysts and enhancers to greatly increase the brightness and stability of light produced by luminol and HRP.

	LUMIBLUE	ECL EXTREME	ECL EXTENDED	ECL EXTRA	ECL PICO	ECL EXPRESS
Signal intensity		Extreme	Very high	High	Medium	Standard
Working solution stability		3 days	3 days	3 days	3 days	5 days
Signal duration		8h	18h	12h	6h	16h
1° Antibody dilutions		1:5,000 – 1:100,000	1:5,000 – 1:50,000	1:1,000 – 1:15,000	1:500 – 1:5,000	1:100 – 1:5,000
2° Antibody dilutions		1:100,000 – 1:500,000	1:50,000 – 1:250,000	1:25,000 – 1:150,000	1:20,000 – 1:100,000	1:1,000 – 1:15,000
Protein quantity		Very-low abundance	Low abundance	Medium abundance	High abundance	High abundance
Protein detection per band		20 pg	150 fg	500 fg	50 fg	2 pg

Unique Technology

Enhanced chemiluminescence (ECL) kits are based on the enzymatic oxidation of luminol with peroxide which produces light, usually through horseradish peroxidase (HRP). LumiBlue uses a patented next generation electron mediator coupled with a novel acylation catalyst to improve performance (called enhancers). These unique modifications increase reaction efficiency which results in increased light intensity and less signal decay.

STRIPPING BUFFER

Our western blot stripping buffer comes as a ready-to-use solution which allows both primary and secondary antibodies to be removed from nitrocellulose/PVDF to allow re-probing of membranes with different antibodies.

- Fast stripping of most antibodies
- Does not damage proteins on blots
- Saves sample by re-probing

PROTEIN MARKERS

RunBlue prestained markers enable easy visualization of the marker proteins. The markers can be used to estimate the molecular weight of your protein of interest and assess the transfer efficiency during blotting.

Expedeon offers two types of prestained molecular weight markers - Tri colour and Dual colour.

BIS-TRIS PROTEIN GELS

RunBlue Bis-Tris gels have superior rigidity and stability over traditional polyacrylamide gels. They give highly comparable results with exceptional reproducibility.

- **Standard Bis-Tris Protocol** – Fast run times at 200V
- **Neutral running pH** – Sharp bands and high protein integrity
- **Unique homogeneous polymerisation** – Increased consistency and no residual free acrylamide
- **Extra Wells** – Run up to 12 or 17 samples simultaneously
- **Deep and wide wells** – Load up to 17 well
- **Protruding teeth** – No well contamination in case of overloading

RUNBLUE FAST BLOTTING BUFFER

RunBlue FAST Blotting Buffer increases the transfer speed of Western blots without excessive heat generation that affects proteins.

- Reduce blotting time by up to 60%
- Smaller protein transfer in less than 10 minutes and larger protein in only 15 minutes



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