



# Western Blot

E-Book



# Overview

Enzo now provides over 40 years of experience in the supply of research kits, biochemicals, and biologics. As *Scientists Enabling Scientists™*, we realize the value in providing relevant information to our customers working in the fields of life sciences, drug development, and clinical research. We are happy to provide simple, but useful tips for improving daily tasks as well as the overall quality of your research.

With this in mind, here is an e-Book that answers important questions for achieving high quality data by Western blot such as:

- Western Blot: Principle and Theory
- Five Western Blot Problems and How to Troubleshoot Them
- How Do You Choose the Right Western Blot Detection Method?
- Membrane Selection: A Quick Comparison of PVDF and Nitrocellulose
- Ten Tips for Successful Westerns

# Western Blot: Principle and Theory

Western blotting is a technique used to separate and identify specific proteins within cell and tissue samples containing a mixture of various proteins. The three main parts of the Western blot technique are **separation of proteins by size, transfer to a solid membrane, and labeling with protein-specific antibodies.**

Western blotting has a few interesting applications, including:

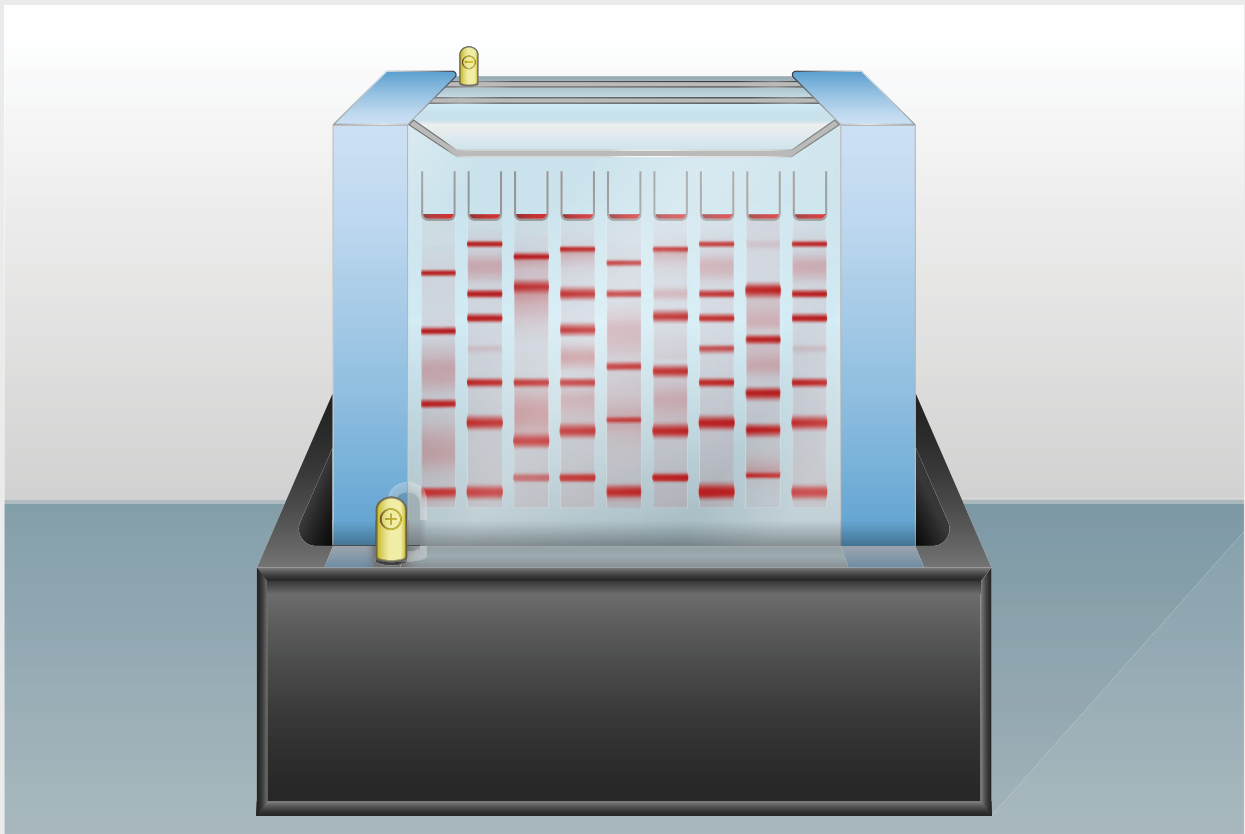
- **Biochemistry:** detection of post-translational modifications in proteins and analysis of protein production
- **Medical diagnostics:** detection of HIV antibodies, Lyme disease, Creutzfeldt-Jakob disease, etc.
- **Enforcing proper practices in the Olympics:** used by the World Anti-Doping Agency to detect blood doping, an illegal technique meant to increase one's red blood cell mass and improve performance

The principle of the Western blot is relatively simple – proteins are first separated by size and then detected using specific antibodies. This technique is an efficient way to confirm protein identity and can be used in conjunction with other detection techniques, such as ELISAs or IHC, to compare protein expression in various tissues or to see how proteins respond to different treatments. Read on to learn more about the main steps involved in this procedure:

- Gel electrophoresis
- Transfer process
- Probing
- Detection

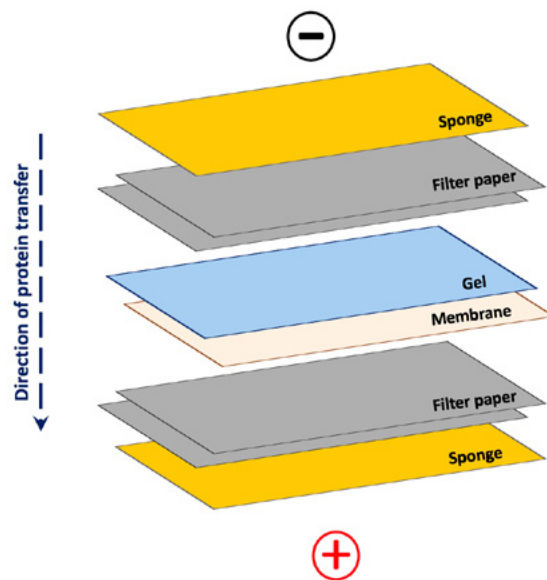
## Gel Electrophoresis

This step is where proteins are separated by their size, or rather, their molecular weight. Often, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) electrophoresis is used for Western blotting: With SDS-PAGE, sodium dodecyl sulfate is used to denature proteins and to grant them a uniform negative charge. Treated proteins are then loaded into the wells of a polyacrylamide together with a marker ladder containing proteins of known molecular weights for comparison. Then, an electrophoresis chamber is used to apply a an electric field voltage across the gel, causing proteins to move toward the positively charged anode. Proteins of low molecular weight travel faster than those of high molecular weight, resulting in proteins separated by size.



## Transfer Process

Once proteins are separated by weight, they are transferred onto a solid membrane. This is usually done by applying an electric field perpendicular to the gel's surface, which draws the proteins out of the gel and onto the membrane. This process, commonly called electro-blotting or short just blotting, can be carried out in different ways. While semi-dry and dry blotting systems become more and more widespread, wet blotting transfer in a liquid tank is still the most widely used technique. To do this, a gel-membrane-fiber "sandwich" is formed. The gel is placed in the middle and the transfer membrane is placed adjacently on what will be the anode side. Very importantly, the gel must maintain close contact with the membrane, with no air bubbles, so that the proteins transfer clearly and uniformly. Then filter paper is placed on both sides, followed by sponges, forming a "sandwich" that will be locked in a support grid structure. The setup is shown in the figure below:



The gel-membrane-fiber sandwich contained in the support grid is then placed into a tank containing transfer buffer and cathode and anode on either side. It is important that the membrane is placed between the gel and the anode, so that the negatively charged proteins will migrate towards the anode and thus get transferred onto the membrane. Most commonly used membranes are either nitrocellulose or polyvinylidene difluoride (PVDF). Both membrane material offer good non-specific protein binding, facilitated by both hydrophobic and charged interactions between proteins and membrane material.

## Probing

Once proteins have been successfully transferred to the solid membrane, they must undergo a blocking procedure to prevent non-specific binding. This is often done with BSA (bovine serum albumin) or non-fat dried milk in TBST (Tris-Buffered Saline Tween-20). Then, the proteins are detected with either one or two antibodies. If direct detection is performed, a primary antibody is used on its own; this antibody specifically binds to the protein of interest. More commonly, indirect detection is used. In this case, a primary and a secondary antibody are used. The secondary antibody is directed against the primary antibody; several secondary antibodies will bind to the primary antibody, thus allowing for an enhanced signal and making it possible to detect proteins at lower concentrations. In either case, once the primary antibody is added and incubated, the membrane is washed with TBST to reduce background and remove any unbound antibody. Similarly, if used, the secondary antibody is then added, incubated, and washed out.

In order for the proteins to be properly visualized, the primary and secondary antibodies must be obtained from separate hosts. If the primary antibody is from a rabbit host, the secondary antibody must be “anti-rabbit” and come from a non-rabbit host.

## Detection



Most commonly, protein detection is performed using secondary antibodies coupled to enzymes. The two main enzymes used for detection in Western blotting are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Chromogenic and chemiluminescent substrates may be added to these enzymes for visualization. Chromogenic substrates react with the enzyme to produce a colored precipitate, yielding results that are visible and readily interpretable to the naked eye. Alternatively, chemiluminescent substrates can be added to induce luminescence, which in turn is detected with the help of photographic film and developers or specialized imaging equipment. Fluorophore-conjugated antibodies may also be used to produce a fluorescent signal that can be detected using specialized equipment.

### References:


<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3456489/>

<https://www.ruf.rice.edu/~bioslabs/studies/sds-page/gellab2.html>

# Five Western Blot Problems and How to Troubleshoot Them




**1 Unusual or Unexpected Bands** If you detect bands at positions lower than expected or are experiencing multiple bands at or below the expected position, there is possible protein degradation occurring. Try to use a newly prepared sample, keep on ice during sample preparation and make sure to add proper and freshly prepared protease inhibitors. If bands are at a higher than expected position, reheat sample to break quaternary protein structure. Alternatively, blurry or not complete bands are usually an indication of a problem with the transfer process. Lowering transfer voltage, keeping the transfer solution cool during the transfer process, and ensuring there are no air gaps between the gel and membrane will help resolve these issues. Also, increasing the acrylamide gel percentage may help with diffuse bands.




**2 No Bands** If you are using a PVDF membrane, remember to activate it by soaking it into methanol prior to use. Make sure to use the appropriate lysis buffer and protocol for sample type and protein of choice. Check that the correct primary antibody and corresponding secondary antibody were used. Also, check the manufacturer's specification sheet to ensure the primary antibody has been validated for Western blots. Next step is to increase the concentration of antibody used or increase the amount of antigen loaded. Additionally, check all buffers for contamination and, when in doubt, make new buffers and new detection reagents. The antigen may also be masked by the blocking buffer or buffers containing azide as preservative may inhibit HRP.

**3 Faint Bands** Similar to the solution for no bands, increase the concentration of the antibody or increase the amount of antigen loaded. Also, if you are using non-fat dry milk as a blocking agent, try using a lower concentration or switch to BSA. Non-fat dry milk may mask the antigen. Alternatively, increasing exposure time or switching to more sensitive detection reagent may enhance the signal.



**4 High Background** This is typically a result of using too high of a concentration of antibody. The antibody will begin to non-specifically bind to the membrane. Increase washing time and add extra washes to decrease background signal. Lowering the exposure time may also help. There may be insufficient blocking of non-specific sites. Compare different blocking buffers. Lastly, decrease the concentration of the primary antibody to reduce non-specific binding.



**5 Patchy or Uneven Spots** This may be related to the transfer process and/or incomplete incubation with the antibodies. Ensure all air gaps between the gel and membrane are removed before the transfer process. Additionally, during incubation with the primary and secondary antibodies, use a rotator or shaker to evenly incubate with the antibody during the incubation periods. When using chemiluminescence make sure to eliminate static charges from your exposure chamber. Lastly, blocking agents may be binding with the antibody. Therefore, switching between nonfat dried milk and BSA may also resolve this issue.

# How Do You Choose the Right Western Blot Detection Method?



**Brian Conrad**

Sales Support Specialist

Due to its flexibility, Western blotting can be used for a wide range of applications within molecular biology and biochemistry, including detection of post-translational modifications and verification of protein cloning. Immobilized membrane-bound proteins are generally detected using secondary antibodies that are conjugated to fluorescent molecules (fluorophores) or an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP). When the enzyme substrate is added, either a colored precipitate (colorimetric detection) or a chemiluminescent or fluorescent product is formed and the light signal is captured. Using the size and color intensity of the protein band, a semi-quantitative estimation of protein can be derived. The new [Chromogenic Western Blot Kit](#) allows for quick, visual reading of Western blots in unpurified cell extract. The kit can be used to identify primary mouse antibodies on a Western blot using immunodetection. It contains a complete reagent set, with optimized ready-to-use or ready-to-dilute reagents.

## Chemiluminescence Detection

Chemiluminescent detection systems take advantage of energy released in reporter enzyme-substrate reactions in the form of light to be the recorded signal. The two commonly used enzyme reporters are HRP and AP. The choice of substrate for chemiluminescent Western blotting is determined by which reported enzyme is chosen. If utilizing HRP, luminol-based reagents are selected. HRP in a peroxide buffer oxidizes luminol to its excited state product, 3-aminophthalate, which emits a light signal at 425nm. When utilizing AP, 1, 2-dioxetane-based reagents are the adjacent AP substrates. AP dephosphorylates 1, 2-dioxetane-based substrates to yield a dioxetane phenolate anion

that emits a light signal at 466nm. These light signals are then captured on X-ray film or charge-coupled device (CCD) cameras as the light signal decays to the ground state. Chemiluminescent systems are easily adapted to traditional Western blotting protocols due to the use of enzyme-conjugated antibodies to activate the light signal. Once an end-user has optimized the correct concentration of enzyme-conjugated antibody, a transient chemiluminescent signal is produced that decays quickly upon exhaustion of substrate. Both methods are compatible on nitrocellulose or PVDF membrane blots.

One of the primary advantages of chemiluminescent Western blot systems is the high degree of sensitivity with respect to protein detection. HRP-substrate reactions can detect down to 1 to 3pg and AP-substrate reactions can detect as little as 10pg of protein. Given the short lived nature of chemiluminescent signals, enhancer chemiluminescent substrates (ECL) have been developed which enable increased signal duration, intensity and sensitivity. With ECL substrates, HRP-substrate systems have an added advantage of being able to detect proteins as low as the femtogram ( $10^{-15}$ ) range.

Chemiluminescent-based methods offer much higher sensitivity relative to colorimetric-based methods that detect proteins within the 100pg-500pg range or fluorescent-based methods which are limited to the nanogram ( $10^{-9}$ ) range. With that being said, higher sensitivity can be disadvantageous in samples that present high background and the end-user must still examine how the affinity of the protein, primary antibody, secondary antibody, and reporter enzyme-substrate will vary in performance from one sample to another and make appropriate optimizations.

### **Do You Need Help?**

Do not hesitate to get in touch with our Technical Support experts. They are here to help!

Visit our website:

[www.enzolifesciences.com/support](http://www.enzolifesciences.com/support),

or email:

[techserv-usa@enzolifesciences.com](mailto:techserv-usa@enzolifesciences.com)

The second key advantage of chemiluminescent systems is the speed at which a protein can be detected. Signals captured with film or CCD imaging cameras offer short exposure times that are 30 seconds to 5 minutes depending on if you use HRP or AP, respectively. Traditionally, film is used to detect chemiluminescent signal and offers the advantages of a large dynamic range compared to other methods, no expensive instrumentation and the retention of an accurate signal-to-noise ratio even with overexposure. However, films

are limited to one-time use and must be developed before one can know whether the exposure time was adequate or not which can lead to frustrating trial and error. Modern CCD imaging systems allow for higher dynamic range and a variety of different exposures, thus enabling the end user to analyze a broad spectrum of protein concentrations within a given sample. Image processing algorithms not only allow for clear specific bands and low background, but also easy semi-quantitative analysis of the results.

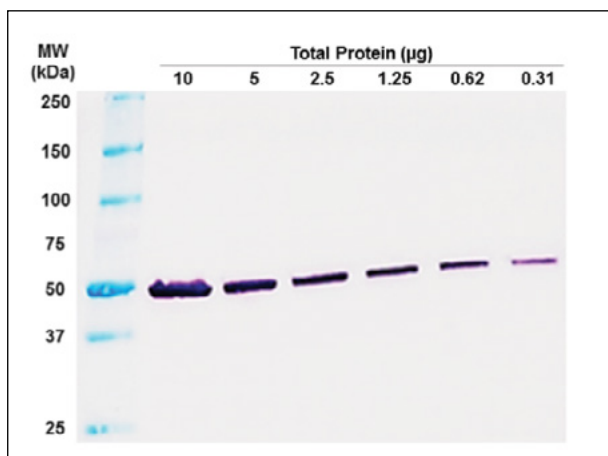
## Chromogenic Detection

Unlike chemiluminescent or fluorescent blotting applications, chromogenic substrates do not require special imaging equipment for visualization of the assay results. Chromogenic detection is an economical and far less labor intensive method for the detection of target protein during a Western blot protocol. Much like chemiluminescent detection, chromogenic detection uses a secondary antibody conjugated to a reporter enzyme that is either HRP or AP. Where chromogenic detection departs from chemiluminescent detection is in the way detectable signal is produced. Instead of reacting with a chemiluminescent substrate, reporter enzymes will react with a soluble, chromogenic substrate to produce a colored, insoluble product which is the detectable signal. This signal precipitates directly onto the blotting membrane to produce colored bands that can be seen by the eye and inform the end-user of the presence or absence of a protein of interest. Similar to film development, the blot is incubated until the desired amount of development is achieved which requires some trial and error to optimize signal intensity. Initially, an end-user must be wary of prolonged incubation as it can increase background signal which could obscure the detectable signal. However, the enzymatic reaction can be visually monitored so the reaction can be stopped with relative ease. This feature makes chromogenic detection a medium-sensitivity technique that is unsuitable for samples with low quantities of the protein of interest.

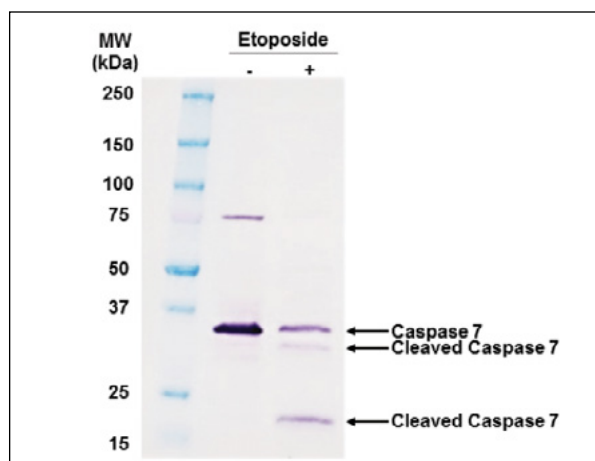
For chromogenic HRP systems, the typical chromogen substrates are 3,3',5,5'-tetramethylbenzidine (TMB), chloro-1-naphthol (4CN) and 3, 3'-diaminobenzidine (DAB). TMB reacts with HRP in a peroxide buffer to produce a water-soluble blue product that is suited for applications that require a large signal to noise ratio. 4CN produces a distinct blue-purple product that is useful for double staining despite the lack of sensitivity or stability afforded by TMB and DAB. DAB yields a brown colored precipitate. The color produced by DAB is intensified by adding metals (nickel, copper, silver, and cobalt) that produce complexes that result in darker colored product and thus, enhance the sensitivity in staining.

For chromogenic AP systems, the typical chromogen substrates are nitro blue tetrazolium (NBT) or 5-bromo-4-chloro-indolyl phosphate (BCIP). NBT is a heterocyclic tetrazolium salt that reduces to yield NBT-formazan, an intensely colored, water-insoluble product that is stable. Therefore, the reaction product will not fade as compared to chromogenic HRP enzyme-substrate products which can fade with exposure to light. NBT is hydrolyzed by AP and produces a blue-purple precipitate that is deposited on nitrocellulose membranes. Unlike chromogenic HRP systems, AP reactions can be inactivated with acidic solutions allowing for multiple probing of the same membrane with alternatively colored antibody probes. AP systems compared to HRP systems will not require such intense chemical stripping that can make the membrane difficult to re-probe for other targets. Ideally though, chromogenic Western blots with AP use a combination of NBT and BCIP that result in an intense, black-purple precipitate which provides a greater substrate sensitivity. NBT/BCIP substrate combinations with AP produce sharp bands with little background staining which is ideal for Western blot application.

The [Chromogenic Western Blot Kit](#) takes advantage of the resource and time efficiency and simplicity of a chromogenic format and the enhanced sensitivity and flexibility of an AP system to provide end-users with highly sensitive and flexible immunodetection through an already optimized, ready-to-use NBT/BCIP solution that is used with anti-mouse secondary antibody conjugated to reporter enzyme. The kit comes with antibody blocker/diluents and wash buffers that are compatible with mouse primary antibodies. During product validation, we have validated assay times of 2 hours with NBT/BCIP exposures times of as little as a minute with low abundance protein targets in Jurkat cell lysates (**Figures 1 and 2**).

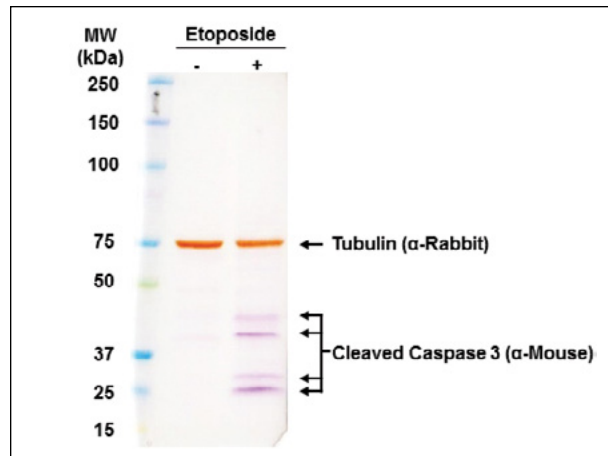


**Figure 1.** Visual Reading for Rapid Results. Total cell lysate from Jurkat cells. Exposure to NBT/BCIP for 1 minute.



**Figure 2.** Detect Difficult Proteins. Jurkat cells treated with 12.5 µM etoposide for 18 hours. Loaded 12 µg lysate per well.

Furthermore, this kit utilizes an AP system that affords end-users the opportunity to multiplex for multiple targets of interest in a single sample so long as the end-user selects a different colored substrate (**Figure 3**).



**Figure 3.** Adaptable to Multiplex Analysis. PANC-1 cells treated with Etoposide. 20  $\mu$ g of total cell lysate loaded. Tubulin labeled with DAB. Caspase labeled with NBT/BCIP.

## Fluorescent Detection

Much like enzyme detection systems, fluorescent detection still uses antigen-antibody complex's to detect specific proteins that have been immobilized on a blot membrane after separation by gel electrophoresis. Fluorescent Western blot detection differs from chemiluminescent detection in the use of fluorochrome-coupled secondary antibodies. The resulting signal is detected with a fluorescence imaging system similar to epifluorescence microscopy. The fluorochrome bound to the antibody is excited with a light source, and the emitted fluorescent signal is detected with the use of wavelength-specific filters and digital camera systems.

A key feature that separates fluorescent detection from enzyme detection systems is that it is more quantitative. Chromogenic or chemiluminescent detection provides a researcher with a semi-quantitative method because the end-user is able to determine the presence or absence of their protein of interest in their samples and through comparison between samples you can infer the expression level of a protein. But with fluorescent detection, the amount of fluorescence produced by a signal is directly proportional to the amount of protein present. However, as in other methods, the end-user still needs to optimize the signal-to-background ratio. Initially, important fluorophore characteristics need to be considered in both fluorophore selection and signal optimization such as the efficiency of photon emission after absorption of light-source photon (quantum yield) and how well a fluorophore absorbs light at a specific wavelength (extinction coefficient). Collectively, these will indicate the relative brightness of the fluorophore.

Fluorophores with higher quantum yields and extinction coefficient are generally brighter. In situations where the end-user is expecting lower-abundance targets in the sample, high initial brightness could be helpful for detection. The difference in maximum excitation and emission wavelengths of a fluorophore (Stokes shift) should be considered for signal optimization as well. Fluorophores with larger Stokes shifts can minimize overlap between excitation and emission wavelengths while increasing the signal intensity. Additionally, the degree of labeling of the secondary antibody with fluorophore may need to be considered. Too little fluorescent labeling and the fluorescence intensity will generate a weak signal. Low signal can also be due to low antigen or excessive washing of the blotting membrane, even if a secondary antibody is used to amplify the signal. Too much fluorescent labeling and the signal will be weak because the detection reagent will be inactivated and the signal will quench due to Förster Resonance Energy Transfer (FRET). To mitigate this, far-red and infrared dye conjugates such as Alexa Fluor 680 and 790 are sometimes used because they offer low quenching, low background fluorescence with high extinction coefficient producing a sensitive, robust signal. Last but not least, the excitation and emission spectra of each fluorophore should be considered in multiplex Westerns to avoid overlap.

Fluorescent Western blot affords several advantages over other methods. For example, the ability to easily multiplex or use several, different colored fluorophores to probe for and detect multiple proteins on the same blot simultaneously. The different colored fluorophores are used to differentiate between target proteins. Unlike enzyme-based methods, there is no need to chemically strip and re-probe the blot when looking at multiple targets. Additionally, the end-user does not need to consider the impact of substrate incubation time or film exposure on detecting a clear signal. However, the end-user does need to consider the potential for cross-reactivity and use primary antibodies from different host species and secondary antibodies that are cross-absorbed against other species. Another advantage comes from the stability of fluorophores. Signals are stable for weeks to months, allowing blots to be stored for re-imaging at a later time point without significant loss of signal.

	Fluorescent Detection	Chemiluminescent Detection	Chromogenic Detection
<b>Substrates</b>	Fluorescent dyes conjugated to secondary antibodies	AP and HRP substrates	Color detection reagents
<b>Detection</b>	Imaging instruments with appropriate filters or lasers	Film and developer	No instrumentation required
<b>Signal Duration</b>	Weeks	Hours	Months
<b>Considerations</b>	Can simultaneously detect multiple proteins on the same blot	Excellent sensitivity with a wide variety of substrates available	Direct visualization with no imaging or film processing instrumentation required

**Table 1.** Western Blot Detection Techniques.



# Membrane Selection: A Quick Comparison of PVDF and Nitrocellulose

The two most commonly used membranes in Western blotting applications are Polyvinylidene fluoride (PVDF) and nitrocellulose. When looking at a new target, it is a good idea to test each type of membrane to determine optimal conditions. In general, nitrocellulose is used for low molecular weight proteins and nucleic acid analysis. Alternatively, PVDF is suitable for higher molecular weight proteins and more durable if strip and re-probing the membrane is necessary. Here are is a comparison of PVDF and nitrocellulose.

Membrane Type	Polyvinylidene difluoride (PVDF)	Nitrocellulose
Protein Binding Capacity	100-300 µg/cm <sup>2</sup>	80-100 µg/cm <sup>2</sup>
Solvent Resistant	Yes	No
Physical Characteristics	Durable	Fragile
Background Noise	Higher sensitivity and background	Lower sensitivity and background
Total Protein Stain Compatibility	Amido black Ponceau S Colloidal gold Colloidal silver India ink Coomassie blue	Amido black Ponceau S Colloidal gold Colloidal silver India ink
Double-blotting Method	Yes	No
Strip and Re-probe	Yes	Possible, but may lose sensitivity
Detection Methods	Chromogenic Chemiluminescent Fluorescent Radioactive Chemifluorescent	Chromogenic Chemiluminescent Fluorescent Radioactive
Other Applications	Amino Acid Analysis Protein Sequencing Solid Phase Assay Systems	Amino Acid Analysis

# Ten Tips for Successful Westerns



**Morgan Mathieu**

Applications Scientist Manager

## 1. Avoid Protein Degradation

Samples should always be prepared quickly, cooled on ice and pan-protease and phosphatase inhibitors added to avoid degradation of proteins. Be aware that commonly used protease inhibitors such as phenylmethanesulfonylfluoride (PMSF) may have short half-lives and need to be re-supplemented with time.

## 2. Prepare Your Samples Appropriately

In general, samples should be boiled five minutes in sample buffer of choice, centrifuged and only the supernatant should be used for SDS-PAGE analysis. If gels are run under denaturing conditions, the actual concentration in reducing agent (e.g. dithiothreitol (DTT) or  $\beta$ -mercaptoethanol) in your sample buffer will need to be high enough to effectively break-up all proteinaceous disulfide bonds. If you are uncertain whether your sample buffer still contains enough reducing agent (NOTE: its effective concentration will decrease with time!), simply add extra. It will do no harm to your samples and you will be on the safe side.



### 3. Boiling vs. Non-boiling of Samples

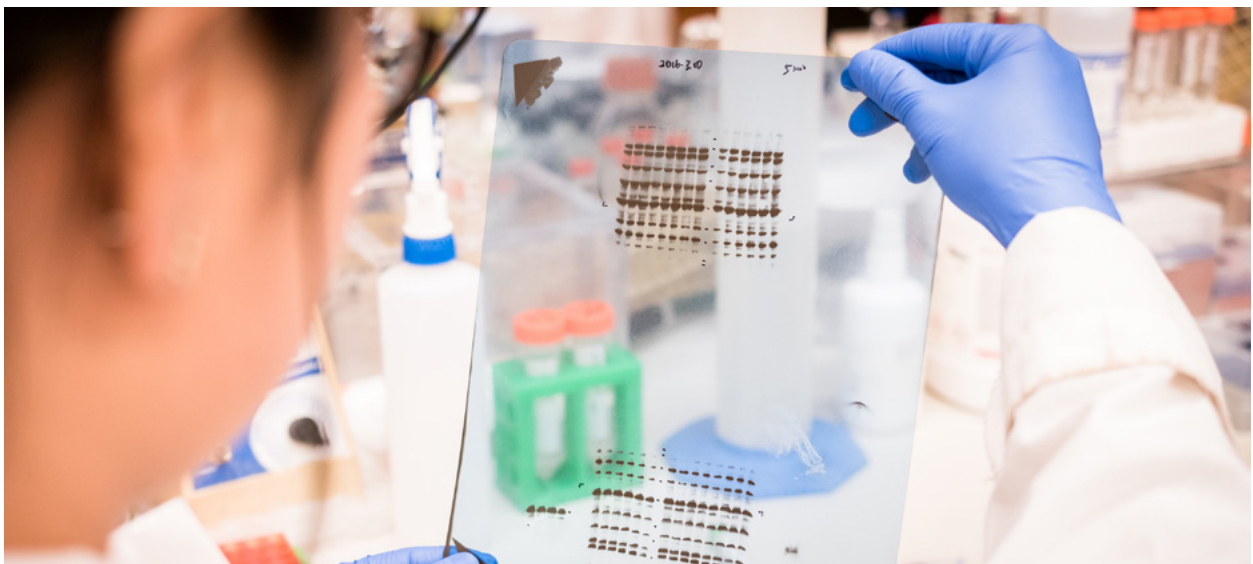
Be aware that highly glycosylated or hydrophobic proteins such as multi-drug resistance (MDR) proteins may precipitate upon boiling. In those instances, you should consider incubating your samples with sample buffer at 60 °C for one hour instead of boiling for five minutes.

### 4. Denaturing vs. Blue/Native Gels

The standard SDS-PAGE for subsequent Western blot analysis is being done under reducing and denaturing conditions. In some cases, this treatment may, however, disrupt the conformation of a three-dimensional epitope that is recognized by a monoclonal antibody. In those instances, a blue/native gel needs to be run. Be aware that due to retaining its three-dimensional structure, your target protein will show a higher apparent molecular weight (MW) in blue/native gels than under standard denaturing conditions.

### 5. Adapt Gel to Match the Requirements of Your Target Protein

Choose the type of gel and its polyacrylamide (PA) content depending on the MW of your target protein and the required accuracy of the MW determination. Standard SDS-PAGE gels range from 7 to 15% PA. To get a good resolution, lower and lower concentrations of PA should be used as the MW of target proteins are getting higher and higher. Especially for complex protein mixtures that are spanning a high MW range, you may need to deploy gradient gels for best resolution. Note that the standard SDS-PAGE using Laemmli glycine-based buffers has a minimum resolution of approximately 16kDa. Hence, for smaller proteins and peptides, alternative methods such as the one developed by [Schägger and von Jagow \(1987\)](#) may be necessary.



## 6. Transfer of High MW Proteins

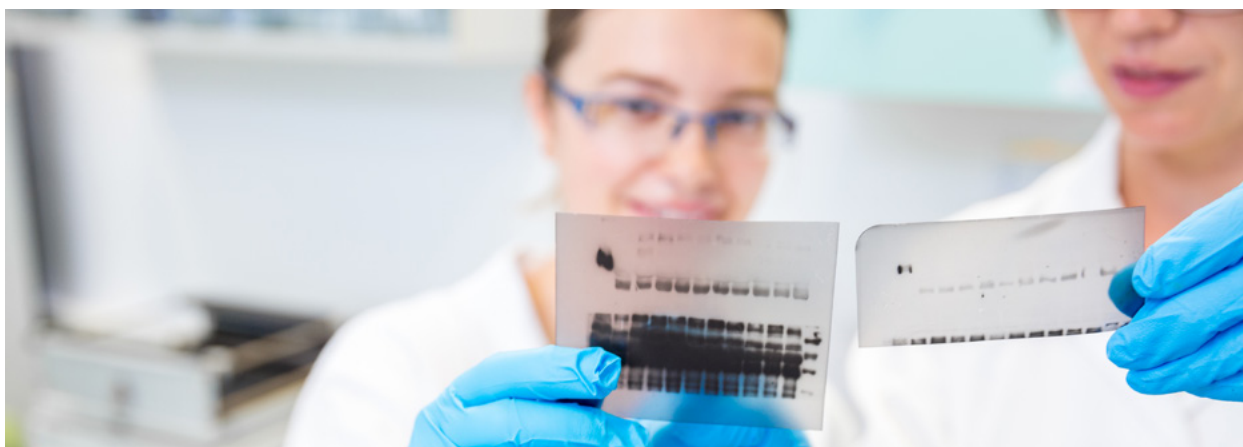
Please note that high MW proteins (above 100kDa) generally transfer more efficiently overnight in a wet transfer apparatus with the addition of SDS in the transfer buffer. Also, it may be necessary to eliminate methanol from the transfer buffer as methanol inhibits elution of high MW proteins out of the gel matrix. It is recommended to use a PVDF membrane instead of nitrocellulose for high MW proteins. Also, use Ponceau S to make sure proteins were transferred from the gel efficiently onto the membrane and stain the gel with Coomassie Brilliant Blue.

## 7. Transfer of Proteins with High Isoelectric Point (pI)

To transfer a protein from gel to membrane, pH of the transfer buffer must be higher than the pI of the respective protein, since only then migration towards the positively charged anode during electrophoresis can occur and transfer to the membrane can happen. For proteins with a pI equal or higher than the pH of the transfer buffer (usually around 8.3), SDS can be included into the transfer buffer to add negative charges to the proteins. In addition, a semi-dry transfer system may need to be used.

## 8. Do Not Deem an Antibody to be Unspecific Until You Have Efficiently Reduced Background

Be aware that not every antibody and sample type will directly match your established Western blot protocol and give you a nice clean blot at first shot. There are a plethora of parameters that may lead to background staining, which may easily be mixed-up with the unspecific staining of an antibody. By careful assay optimization, you should almost always be able to substantially reduce background and get a nice clear signal in your Western blots. The use of appropriate controls is essential in this optimization process.



## 9. Check That the Blocking Procedure is Appropriate for Your Target Protein

The standard blocking agent in Western blot is non-fat dry milk. However, performance of some antibodies may be significantly adversely affected by blocking with milk or casein, since the antigens/epitopes they detect may be present in those blocking agents at concentrations that can be high enough to elevate the background of a blot and obscure a positive signal. Therefore, use of milk or casein should always be avoided for, e.g., antibodies to Ubiquitin, phosphoserine or phosphothreonine. In those instances, always use serum of the secondary antibody host or BSA for blocking and for incubation with the primary antibody.

## 10. Always Use Well-established Controls to Validate Your Assays

It is crucial to validate your Western blots with reliable positive and negative controls. Particularly when looking at endogenous proteins produced in low amounts, even a good positive control lysate may require input of up to 150µg of total protein per lane. Also, cells transfected with your target protein or the purified (recombinant) protein may serve as a good source for positive controls. As a negative control, you should always run a “secondary antibody only” blot in parallel to assess how much background actually stems from the secondary antibody and detection system deployed. If available, the immunogenic peptide an antibody was raised against can also be used as a blocking peptide and be very helpful in distinguishing the true signals of your target protein from background staining.

Although these are some tips, there are clearly many other issues that need to be considered and addressed in order to ensure a successful experiment, such as washing buffer, antibody concentration, secondary antibody etc. However, the key thing is to understand the principles and practice of Western blotting, and be mindful of the drawbacks and boundaries of this approach for protein analysis.

### Did You Know?

The Southern blot is named after British biologist Edwin Southern. All other blotting methods such as Western blots, Northern blots, and Eastern blots are named in reference to Southern's name.



**Global Headquarters**  
**ENZO LIFE SCIENCES, INC.**  
10 Executive Blvd.  
Farmingdale, NY 11735  
Ph: 800.942.0430  
Fax: 631.694.7501  
info-usa@enzolifesciences.com

**European Sales Office**  
**ENZO LIFE SCIENCES (ELS) AG**  
Industriestrasse 17  
CH-4415 Lausen, Switzerland  
Ph: +41 61 926 8989  
Fax: +41 61 926 8979  
info-eu@enzolifesciences.com

#### **LOCAL EUROPEAN OFFICES**

**Belgium, The Netherlands & Luxembourg**

Enzo Life Sciences BVBA  
Avenue Louise 65/Box 11  
1050 Bruxelles  
Belgium  
Ph: +32 3 466 0420  
Fax: +32 3 808 7033  
info-be@enzolifesciences.com

**France**

Enzo Life Sciences (ELS) AG  
Branch Office Lyon  
13, avenue Albert Einstein,  
F-69100 Villeurbanne, France  
Ph: +33 472 440 655  
Fax: +33 481 680 254  
info-fr@enzolifesciences.com

**Germany**

Enzo Life Sciences GmbH  
Basler Strasse 57a  
DE-79540 Lörrach  
Germany  
Ph: +49 7621 5500 526  
Fax: +49 7621 5500 527  
info-de@enzolifesciences.com

**UK & Ireland**

Enzo Life Sciences (UK) Ltd.  
1 Colleton Crescent  
Exeter EX2 4DG  
Ph: 0845 601 1488 (UK customers)  
Ph: +44 1392 825900  
Fax: +44 1392 825910  
info-uk@enzolifesciences.com

For local distributors and detailed product information visit us online:

**[www.enzolifesciences.com](http://www.enzolifesciences.com)**

**Put our experience to work for you!**

Our broad range of scientific expertise and manufacturing capabilities enables us to provide innovative tools to save you time and money!