Chemiluminescence western blotting technical guide and protocols

Introduction

Western blotting is a powerful and commonly used tool to identify and quantify a specific protein in a complex mixture. As originally conceived by Towbin et al., the technique enables indirect detection of protein samples immobilized on a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. In a conventional western blot, protein samples are first resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to the membrane. Following a blocking step, the membrane is probed with a primary antibody (polyclonal or monoclonal) that was raised against the antigen in question. After a subsequent washing step, the membrane is incubated with a secondary antibody conjugated to an enzyme that is reactive toward the primary antibody. An enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) is necessary for signal generation. After a final wash step, the membrane is incubated with an appropriate enzyme substrate to generate a recordable signal.

This guide describes general principles and considerations involved in western blotting using chemiluminescent substrates. Relevant Thermo Scientific™ and Invitrogen™ products in protein research are referenced throughout, but the strategies and methods discussed are applicable to most enhanced chemiluminescence (ECL) western blot assays.

Types and considerations of chemiluminescence western blotting

HRP is the most popular enzyme used in western blotting and will be discussed throughout this document as our example. The most suitable western blotting substrates for HRP are luminol-based, and they produce a chemiluminescent signal. Chemiluminescence is a chemical reaction that produces energy released in the form of light. In the presence of HRP and a peroxide buffer, luminol oxidizes and forms an excited-state product that emits light as it decays to the ground state. Light emission occurs only during the enzyme–substrate reaction and, therefore, once the substrate in proximity to the enzyme is exhausted, signal output ceases. In contrast, colorimetric substrates, such as diaminobenzidine (DAB), produce precipitate that remains visible on the membrane even after the reaction has terminated. Several varieties of Thermo Scientific™ Pierce™ ECL and SuperSignal™ chemiluminescent HRP substrates are available that provide different levels of sensitivity for chemiluminescence western blotting. Refer to Table 1 to select the most appropriate HRP chemiluminescent substrate based on the abundance of your target protein of interest, abundance of sample containing the target protein, and the level of sensitivity and type of instrumentation available for detection.

Table 1. Recommended Pierce ECL and SuperSignal chemiluminescent substrates.

	Pierce™ ECL substrate	Pierce ECL™ Plus substrate	SuperSignal™ West Pico PLUS substrate	SuperSignal™ West Dura substrate	SuperSignal™ West Femto substrate
Detection level	Low- to mid-picogram	Low-picogram	Low-picogram to high-femtogram	Mid-femtogram	Low- to mid-femtogram
Signal duration	0.5–2 hr	5 hr	6–24 hr	24 hr	8 hr
Detection methods	X-ray film, CCD imager	X-ray film, CCD imager, fluorescence imager	X-ray film, CCD imager	X-ray film, CCD imager	X-ray film, CCD imager
Select when …	… target and sample are abundant	… target is less abundant, sample is limited, and you need chemifluorescent detection	… target is less abundant, sample is limited, and you need more sensitivity than an entry-level ECL substrate provides	… target is less abundant, sample is limited, and you are using CCD image capture	… target is least abundant, sample is precious, and you need maximum sensitivity
Value to you	Low cost; easy to switch from other entry-level ECL substrates	Best detection flexibility with chemifluorescent detection option	Best value; works for majority of western blots	Best signal duration	Best sensitivity

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Signal capture

Although western blotting is a powerful, routine application, capturing that elusive chemiluminescent signal can be frustrating. Since western blotting comprises a series of steps that require specific skills to perform, failure to capture signal can be caused by many factors. With so many variables (Table 2), troubleshooting a problem blot can be likened to searching for a "needle in a haystack". The traditional protocol is often ineffective in detecting a specific protein. For example, the primary antibody may not recognize the immobilized antigen in its denatured state. Although the protein can be kept in its native state by using nondenaturing conditions, this makes determination of the target molecular weight more challenging. Further, exceptionally large or hydrophobic proteins are often difficult to transfer. Towbin's original western blot protocol is therefore often modified to ensure target detection. Such modifications may involve the use of different reagents for indirect detection or a labeled primary probe for direct detection of the target. Sometimes transfer of protein from a gel to membrane is bypassed altogether and detection is accomplished in the gel.

Table 2. Factors that affect western blotting results.

Factor	Variable characteristic
Target antigen	Conformation, stability, available epitope(s)
Polyacrylamide gel	Manufacturer, percent polyacrylamide, age, lot
Membrane	Manufacturer, type, lot
Primary antibody	Specificity, titer, affinity, incubation time and temperature
HRP conjugate	Enzyme activation level and activity, source animal, concentration, detergent
Blocking buffer	Type, concentration, cross-reactivity
Washes	Buffer, volume, duration, frequency
Substrate	Sensitivity, manufacturer lot, age
Detection method	Film age, imaging instrument manufacturer, exposure time

Signal intensity and duration

Under optimal western blotting conditions, a chemiluminescent signal can last for 6–24 hr. The level and duration of light generation depends on the specific substrate being used and the enzyme-to-substrate ratio in the system. Although the amount of substrate on a blot is relatively constant, the amount of enzyme present depends on the amount added and other factors (Table 2). Too much enzyme conjugate applied to a western blot system is one of the greatest cause of signal variability, dark background, short signal duration, and low sensitivity.

A signal emission curve that decays slowly (Figure 1) is desirable as it demonstrates that each component of the system has been optimized and allows reproducible results. A signal that decays too quickly can cause variability, low sensitivity, high background, and problems with signal documentation. A long-lasting signal minimizes variability in results due to transfer efficiency, different manufacturer lots of substrate, and other factors.

The oxidation reaction of the HRP molecules with the luminol in the substrate produces free radicals in addition to the light being produced. An abundance of HRP in the system will create an abundance of free radicals, speeding the probability of HRP inactivation. Free radicals may also damage the antigen, antibodies, and the membrane, resulting in reduced effectiveness of re-probing.

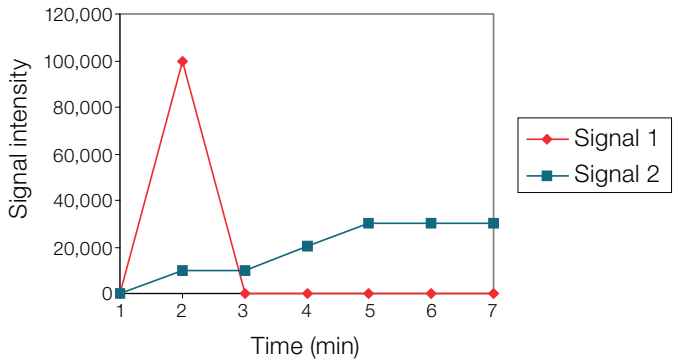

Figure 1. A comparison of signal emission curves for short- and long-duration substrates. When there is an enzyme present in a western blot system, signal output peaks soon after substrate application and rapidly exhausts the substrate (signal 1). In an optimal system, the signal emission peaks approximately 5 min after applying the substrate and plateaus for several hours (signal 2).

Direct and indirect methods

Direct detection uses a labeled primary antibody. Because incubation with a secondary antibody is eliminated, this strategy takes less time than a classic western blot. Additionally, background signal from cross-reactivity with the secondary antibody is eliminated. Direct detection also enables probing for multiple targets simultaneously. Labeling a primary antibody, however, sometimes has an adverse effect on its immunoreactivity, and allows for no signal amplification. As the direct method is generally less sensitive than an indirect detection, it is most useful when the target is relatively abundant. One option is biotinylating the primary antibody, which is an indirect method that both amplifies the signal and eliminates the need for a secondary antibody. Labeling with biotinylation reagents typically results in more than one biotin moiety per antibody molecule. Each biotin moiety is capable of interacting with enzyme-conjugated avidin, streptavidin, or Thermo Scientific™ NeutrAvidin Protein (**Cat. No. 31000**). Essentially, the biotin-binding conjugate replaces a secondary antibody, and its appropriate molar concentration is the same as if a secondary antibody were used. The small size of biotinylation reagents (typically less than 2 kDa) compared to enzymes (40 kDa for HRP; 140 kDa for AP) often reduces steric hindrance, allowing greater functionality of the labeled primary antibody.

Far-western methods

Occasionally, an antibody to a specific antigen is unavailable or unsuitable for western blot analysis. Target protein–specific detection by blotting is also possible if a corresponding binding partner is available for use as a probe. This type of application, referred to as a far-western blot, is routinely used for the discovery or confirmation of a protein–protein interaction. There are numerous variations in western blotting detection that include, but are not limited to, the previously mentioned strategies. In far-western blotting, labeled binding partners may be labeled by an *in vitro* translation reaction with ^{35}S-labeled probe. Biotinylating the probe and detecting with a biotin-binding protein conjugate is also possible. This type of detection has the added advantage of signal amplification. However, when using this technique the probe should not be over-labeled, to prevent target detection from being compromised. Additionally, a recombinant probe can be expressed in bacteria with a tag such as GST, HA, c-Myc, or FLAG, in which detection occurs via a labeled antibody to the particular tag. As with other blotting applications, the far-western method is effective for both membrane and in-gel detection systems.

Optimization

Each western blot system must be optimized to obtain consistently good results. Many factors influence signal intensity and duration, and each of these factors can be optimized. Included below are discussions on several factors. The protocol section at the end of this guide includes specific instructions and suggestions about optimization procedures.

Blotting membrane

HRP–luminol interactions and subsequent signal generation are likely unaffected by membrane composition. Nitrocellulose and PVDF membranes, however, do differ in their protein-binding properties. Generally, PVDF has a higher binding capacity than nitrocellulose, high tensile strength, and excellent handling characteristics. However, PVDF is more hydrophobic, which makes it potentially more difficult to wet and it sometimes results in more background signal compared to other membranes. Since PVDF membranes from different manufacturers behave differently, researchers need to make sure that the PVDF membrane from each manufacturer is optimized within a given system. For best results, empirically test a given membrane prior to optimizing with precious samples or antibodies. Owing to the lot-to-lot differences between membranes, it may be beneficial to test each new lot of membrane.

Protein transfer

Transfer efficiency varies dramatically between different proteins. They differ in their ability to migrate from the gel and their tendency to bind to the membrane using a particular set of conditions. Transfer efficiency depends on factors such as gel composition, the gel-membrane contact, position of the electrodes, transfer duration, protein size and composition, field strength, buffer composition, and the presence of detergents. Optimal transfer of most proteins via wet tank systems is obtained in low–ionic strength buffers and with low electrical current. However, high–ionic strength buffers are necessary for fast transfers that can be performed at high current. Transfer efficiency can be evaluated by staining the membrane with an immunoblot-compatible or reversible stain.

Blocking buffer

Many different blocking reagents are available for western blotting applications. Since there is no one optimum blocking reagent for all systems, empirical testing is essential. An optimal blocking buffer maximizes the signal-to-noise ratio and does not react with the system's antibodies or targets. For example, using 5% nonfat milk as a blocking reagent in avidin–biotin systems results in high background because milk contains variable amounts of endogenous biotin, which binds to biotin-binding proteins. In addition, milk contains phosphatases, which could dephosphorylate the target protein, and interfere with detection of previously phosphorylated molecules in the sample. When switching substrates, antibodies, or the target, a diminished signal or increased background can result simply because the blocking buffer was not optimal for the new system.

Some systems may benefit from adding a surfactant, such as Thermo Scientific™ Tween™ 20 detergent, to the blocking solution. Surfactants can minimize background by preventing the blocking reagent from nonspecifically binding to the target. Adding too much detergent, however, can prevent adequate blocking. Typically, a final concentration of 0.05% detergent is used; however, it may not be required or optimal for all systems. It is recommended that a high-quality detergent such as Thermo Scientific™ Tween™ 20 Surfact-Amps™ Detergent Solution (**Cat. No. 28320**), which is a specially purified Tween 20 detergent free of peroxides and carbonyls, be used. This will prevent these contaminants from interfering with the assay. Thermo Scientific™ StartingBlock™ T20 Blocking Buffer in PBS (**Cat. No. 37579**) or TBS (**Cat. No. 37543**) contains Tween 20 detergent at a concentration of 0.05%.

Antibodies

Western blotting is typically performed by probing the blocked membrane with a primary antibody that recognizes a specific protein or epitope in a complex mixture of proteins. The choice of a primary antibody will depend on the specific antigen being detected and the corresponding available antibodies. It is also important to note that not all primary antibodies are suitable for western blotting. So, antibodies that are validated for this application should only be used. Generally, tagged secondary antibodies are used as the means of ultimately detecting the target antigen (indirect detection). A wide variety of labeled secondary antibodies can be used for western blot detection. The choice of secondary antibody depends on either the species of animal in which the primary antibody was raised (the host species) or any tag linked to the primary antibody (e.g., biotin, multiple histidines (6xHis), hemagglutinin (HA), etc.) For example, if the primary antibody is an unlabeled mouse monoclonal (IgG or IgM) antibody, then the secondary antibody must be an anti–mouse IgG or anti–mouse IgM antibody obtained from a non-mouse host.

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* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

Not only is the affinity of the primary antibody for the antigen important, but the concentrations of primary and secondary antibodies also have profound effects on signal emission. An excessive amount of HRP captured on the blot may be a result of excessive concentration of either the primary or secondary antibody, or both. Minimizing the primary antibody concentration is advantageous, as it promotes target-specific binding and low background. If the signal is inadequate due to high background or low antibody binding, enhancer products can be used as a pretreatment to increase the signal of the western blot. The Thermo Scientific™ SuperSignal™ Western Blot Enhancer (**Cat. No. 46640**) can help improve detection of the target protein. It is a 2-part system that contains a membrane treatment reagent and primary antibody diluent to increase signal intensity and sensitivity, improving overall signal-to-noise ratio in the system (Figure 2).

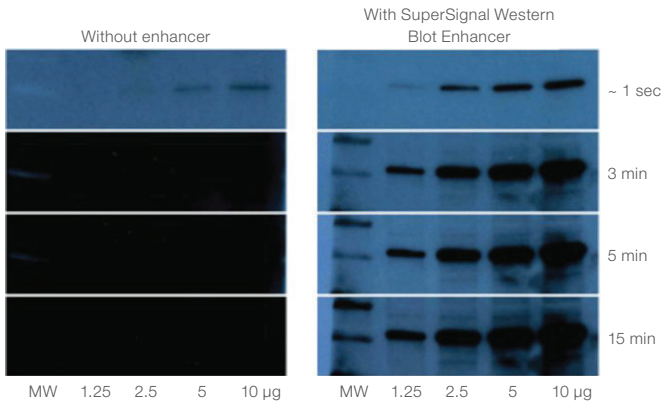

Figure 2. SuperSignal Western Blot Enhancer reduces background to enable detection of low-abundance targets. K562 cell lysate was loaded into Tris-glycine SDS-PAGE gels at 1.25, 2.5, 5, and 10 µg per lane. (Left) After electrophoresis, the proteins were transferred to nitrocellulose membranes (Cat. No. 88013) and the membranes were blocked with Thermo Scientific™ SuperBlock™ Blocking Buffer (Cat. No. 37535). The blot was probed with mouse anti-ERK 1 primary antibody at 1 µg/mL (Cat. No. MA1-13041) and then a horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG (Cat. No. 31430) secondary antibody at 0.08 µg/mL. Thermo Scientific™ Pierce™ ECL Substrate (Cat. No. 32209) was used for detection. Both the primary antibody and secondary antibody conjugates were diluted in blocking buffer. (Right) The protocol is same as described above except this protocol included use of SuperSignal Western Blot Enhancer. The membranes were pretreated with the Antigen Pretreatment Solution for 10 min, rinsed with water, and incubated with the blocking buffer. The primary antibody was diluted in Primary Antibody Diluent prior to probing the membrane.

If a blot fails to generate an adequate signal, remove all the detection reagents from the blot and reprobe with either a different primary antibody at a fixed concentration or different concentrations of the antibody. This often conserves valuable sample and time; however, insufficient stripping can leave active HRP on the blot that can also produce signal. To check for active HRP remains on a stripped blot, reapply the substrate and check for signal. Also, an abundance of inactive HRP molecules not removed by stripping may inhibit the primary antibody from binding to the target on reprobing. Stripping and reprobing blots is an effective method to gain more information about a specific system and conserves valuable samples.

Detection method

Traditionally, to detect a western blot chemiluminescence signal, film is used. This film requires no expensive equipment and provides excellent sensitivity. Unfortunately, each piece of film can only be used once and must be developed before determining whether or not the exposure time was appropriate. Trial and error (i.e., using several sheets of film) is often required. Often, it is necessary to expose several pieces of film for different time periods to obtain the proper balance between the signal and background. If the film is overexposed, the signal may be lost in the background or separate bands may become blurred together. The overexposed film can be "rescued" using reagents such as Thermo Scientific™ Pierce™ Background Eliminator (**Cat. No. 21065**) (Figure 3).

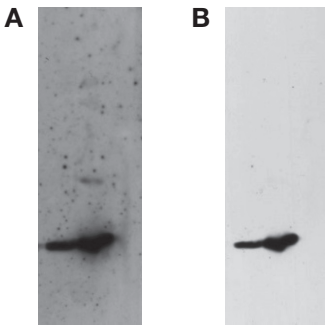

Figure 3. Recombinant human TNFα was separated by SDS-PAGE using a 4–20% gel and transferred to a nitrocellulose membrane. After blocking, the blot was probed with a mouse anti–human TNFα antibody and a goat anti-mouse secondary antibody conjugated to HRP, and detected with SuperSignal West Dura substrate (Cat. No. 34076). The blot was exposed to film for 30 sec, resulting in considerable background speckling **(A)**. The film was then treated with Thermo Scientific™ Pierce™ Background Eliminator (Cat. No. 21065) for 2 min to remove speckling and band overexposure **(B)**.

Charge-coupled device (CCD) cameras are now commonly used to detect chemiluminescence. These imagers and their accompanying analytical software such as the Invitrogen™ iBright™ CL1000 Imaging System (**Cat. No. A32749**) are capable of adjusting the background signal and performing densitometry. The imager has several advantages over film-based detection. The large dynamic range and the high degree of exposure control allow for the best possible image documentation. Additionally, optimization of exposure time avoids saturation of band signal and allows for observation of minor variations in density. In contrast, film has a smaller dynamic range of detection, and band signals on film can quickly reach saturation. When signal intensity is high, the film's low dynamic range along with its propensity to reach saturation quickly and the limitations of exposure control often result in overexposed images.

Common occurrences and explanations

No signal

An initial exposure that fails to capture the signal in a western blot indicates that the blotting system requires optimization. Frequently, lack of signal is caused by an increased amount of HRP in the system. It may seem counterintuitive to use less enzyme conjugate when a signal cannot be detected; however, for successful signal documentation, an optimal amount of antigen and primary antibody, and the correct balance of enzyme and substrate must be present. Substrate oxidation by the enzyme is irreversible and, therefore, once the substrate is oxidized, it can no longer interact with the enzyme to generate light. Because enzyme activity persists, the substrate is the limiting factor in the reaction. Once the substrate is exhausted, signal output ceases. Rarely, a lack of signal is caused by an insufficient amount of active enzyme present. Too much or not enough enzyme can be caused by any of the factors involved in the western blot system.

To produce a signal that can be captured, adjust the system's parameters. For reproducible results, prepare a new gel, apply an optimal amount of sample, and titrate the primary and secondary antibodies. When optimizing antibody concentrations, image the blot twice—once immediately after substrate incubation and a second time at an interval after incubating the substrate (e.g., 1 hr). The second detection provides information about the optimal enzyme concentration and helps optimize parameters. For example, if imaging the blot initially produced signal but ceases to do so after an hour or other reasonable time interval, the blot conditions might have resulted in excess HRP.

Also, if the initial exposure does not capture a signal, a second, longer incubation with the substrate may yield a signal if there is little active HRP on the blot. Stripping all detection reagents from the blot and reprobing can save valuable sample while optimizing the process parameters. If blot-to-blot consistency is desired, the same conditions must be used and the same procedure must be followed each time the experiment is performed.

Signal fades quickly

When a particular system produces a signal that fades quickly, the western blot requires optimization, as described in the above section. However, obtaining a signal in the western blot indicates that the system is working, and the blotting conditions simply need to be tweaked. A signal that exhibits the desired long duration indicates that the blotting conditions are fully optimized for the system. A successful but suboptimal system is subject to the slight variations inherent to the method, such as transfer efficiency and changes in sample and antibody activity during storage and handling.

New bottle of substrate does not produce a signal

Occasionally, a signal may not be captured when the only variable that has changed in a particular system is the bottle or lot of the substrate used. Typically this result is caused by a western blot system that has not been fully optimized. Western blotting substrates may have minor variation from lot to lot. In a fully optimized blotting system, substrate sensitivity variations, as well as other variables, are minor or unnoticeable.

Brown or yellow bands on the membrane

HRP becomes brown when it is oxidized and inactive, causing most HRP conjugates to have a yellow to brown hue. In an optimized system, the amount of oxidized HRP is minuscule and cannot be visualized on the blot. The appearance of yellow or brown bands indicates presence of a large amount of HRP and therefore, the oxidized and inactive portion is visible. A blotting system that results in yellow bands requires optimization using much less enzyme conjugate. Additionally, a high amount of HRP in a localized area means that the reaction of the HRP with the substrate in that area will result in a highly localized

concentration of free radicals. These free radicals can inactivate HRP and damage antibodies, and can also target the membrane, prohibiting effective reprobing.

Bands or entire blot glowing in the darkroom

If a pattern of bands or the entire blot glows after incubation in the substrate, then there is likely a high amount of HRP present in the system. This occurrence indicates that further dilution of the secondary antibody–HRP conjugate is required and possibly of the primary antibody as well. Presence of an excessive amount of enzyme can be potentially caused by many of the factors involved in the western blotting system. If the entire blot is glowing, optimization of blocking and washing steps, in addition to optimization of sample, primary antibody dilution, and secondary antibody conjugates, may be necessary.

Ghost or hollow bands

A protein band that has a halo in the middle of the band or an entire band that appears white in a dark background are typically referred to as ghost bands. This occurrence indicates depletion of substrate in the white area. The most common causes of ghost band effects are applying too much of the target protein to the gel and using too high concentrations of secondary antibodies.

High background

High background signal is the result of either insufficient blocking, cross-reacting of antibodies and blocking buffer, or using a high amount of enzyme conjugate. It is a common misbelief that a particular substrate can cause background signal or can increase the background signal. In reality, the substrate in itself cannot cause any signal without the enzyme being present. When changing to a substrate with greater sensitivity than what was previously used, high background often results if the parameters, such as antibody concentrations, were not altered to compensate for the substrate's sensitivity. Using optimal concentrations of antibodies promotes target-specific binding and low background.

General protocol for blotting and stripping using chemiluminescent substrates

This section contains in-depth protocols for western blotting, stripping membranes, and optimization. While this guide does not describe every eventuality and aspect of western blotting, it provides an accurate overview of the uses, limitations, and potential of this powerful technique.

We offer a wide selection of precast gels, electrophoresis buffers, blocking and wash buffers, primary and secondary antibodies, chemiluminescent substrates, film, imaging systems, and other essential reagents for western blotting. See the "Related products" section on the last page or visit the website for more information.

Conventional western blotting protocol

1. Separate the proteins in the sample by gel electrophoresis.

2. Prepare the transfer buffer: Use Tris-glycine transfer buffer dissolved in 400 mL of ultrapure water plus 100 mL methanol (25 mM Tris, 192 mM glycine, pH 8.0, 20% methanol). Use and store the transfer buffer at 4°C.

3. Construct a gel sandwich (Figure 4) for wet transfer. For semi-dry transfer, prepare the sandwich in the same order between the anode and cathode.

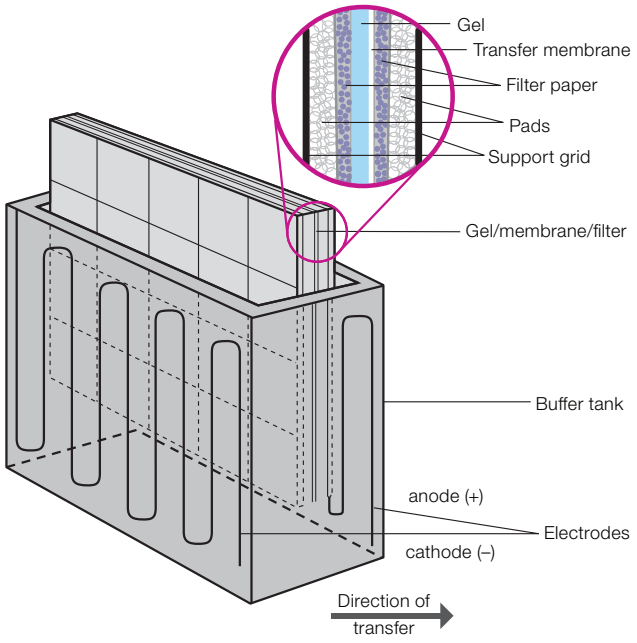

Figure 4. Electrophoretic transfer setup.

4. Transfer proteins from the gel to a membrane. For wet transfer using a mini-transfer apparatus designed for a 8 x 10 cm gel, transfer at 40 V for 90 min, keeping the buffer temperature at 4°C. For semi-dry transfer use 15 V for 90 min.

5. Remove the membrane and block the nonspecific binding sites with a blocking buffer for 20–60 min at room temperature (RT) with shaking.

6. Incubate the blot with the primary antibody solution (Table 3) containing 10% blocking solution with continuous rocking for 1 hr. If desired, incubate the blot overnight at 2–8°C.

7. Wash the membrane three times for 5 min each with Tris-buffered saline (TBS), phosphate-buffered saline (PBS), or other physiological wash buffer containing 0.05% Tween 20 detergent. If using an enzyme-conjugated primary antibody, proceed to Step 10.

8. Incubate blot with the enzyme conjugate (Table 4) containing 10% blocking solution with continuous rocking for 1 hr at RT.

9. Wash the membrane 5 times for 5 min each in wash buffer to remove any unbound secondary antibody conjugate. It is crucial to thoroughly wash the membrane after incubation with the enzyme conjugate.

10. Prepare the substrate. Use a sufficient volume to ensure that the blot is completely wetted with the substrate and the blot does not become dry (0.1 mL/cm^2).

11. Incubate the blot with the substrate for 1 min when using Pierce ECL substrate or 5 min when using SuperSignal substrates.

12. Remove the blot from the substrate and place it in a plastic membrane protector. A plastic sheet protector works well, although a plastic wrap may also be used. Remove all air bubbles between the blot and the surface of the membrane protector.

13. Image the blot using film or a cooled CCD camera.

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- There is a hands-free alternative for all blocking, antibody incubation, and washing steps. Use our Invitrogen™ iBind™ and iBind™ Flex Western Devices. Find out more at **thermofisher.com/ibind**

Western blot stripping protocol

1. Prepare the stripping buffer. Use one of the following suggested stripping buffers:
   - Thermo Scientific™ Restore™ Western Blot Stripping Buffer (**Cat. No. 21059**)
   - Thermo Scientific™ Restore™ Plus Western Blot Stripping Buffer (**Cat. No. 46428**)
   - 0.1 M glycine·HCl (pH 2.5–3.0)
   - 50 mM Tris·HCl (pH 7), 2% SDS, 50 mM dithiothreitol (DTT)
2. Place the blot to be stripped in stripping buffer. Use sufficient volume to ensure that the blot is completely wetted (approximately 20 mL for an 8 x 10 cm blot).

Table 3. Recommended primary antibody dilutions to use with Thermo Scientific™ chemiluminescent substrates.

Substrate	Pierce ECL	Pierce ECL Plus	SuperSignal West Pico PLUS	SuperSignal West Dura	SuperSignal West Femto
Recommended primary antibody dilutions	1:1,000	1:1,000	1:1,000	1:5,000	1:5,000

Table 4. Recommended secondary antibody dilutions to use with Thermo Scientific chemiluminescent substrates.

Substrate	Pierce ECL	Pierce ECL Plus	SuperSignal West Pico PLUS	SuperSignal West Dura	SuperSignal West Femto
Recommended secondary antibody dilutions	1:1,000–1:15,000	1:25,000–1:200,000	1:20,000–1:100,000	1:50,000–1:250,000	1:100,000–1:500,000

3. Incubate for 5–15 min at room temperature. Optimization of both incubation time and temperature is essential for best results. Some interactions require at least 15 min of incubation and may require incubation at 37°C. If using a glycine buffer at low pH, incubate for 30 min at 70°C.

4. Remove the blot from the stripping buffer and wash using wash buffer (PBS/TBS or other physiological buffer containing 0.05% Tween 20 detergent).

5. To test for complete removal of the enzyme conjugate and primary antibody, perform the tests listed below. If a signal is detected in either case, repeat steps 2–4, stripping for an additional 5–15 min or increasing the temperature to 37°C. Optimize stripping time and temperature to ensure complete removal of antibodies while preventing damage to the antigen.

   - To test for complete removal of the enzyme conjugate, incubate the membrane with substrate and image the blot. If no signal is detected after a 5 min exposure to film, the enzyme conjugate has been successfully removed from the membrane.

   - To test for complete removal of the primary antibody, incubate the membrane with enzyme conjugate and then wash with wash buffer. Incubate with the substrate and image the blot. If no signal is detected after a 5 min exposure to film, the primary antibody has been successfully removed from the membrane.

6. After determining that the membrane is properly stripped, commence the second probing experiment. Typically, a blot can be stripped and reprobed several times but may require longer exposure times or a more sensitive substrate after each stripping. Subsequent reprobing may result in decreased signal if the antigen is labile. In such case, analysis of the individual system is required. If you want to probe a protein expressed from a housekeeping gene in order to standardize the protein loading, probe the target protein first to ensure no loss of signal due to stripping of the membrane.

Optimization procedures for chemiluminescence western blotting

Optimizing antigen concentration

1. Prepare different concentrations of the protein sample in SDS-PAGE sample buffer. Test a wide range of sample concentration, keeping in mind the detection limit of the substrate being used.

2. Apply an equal volume of each concentration on the gel and separate the samples by electrophoresis. Transfer the samples to a membrane.

3. Block the membrane with a standard blocking reagent and probe with the primary antibody followed by the enzyme conjugate. If optimized dilutions have not yet been determined, use a mid-range value according to the sensitivity of the substrate.

4. Wash membrane and add the substrate. Image the blot as desired.

Optimizing membrane blocking

1. Separate the protein sample by electrophoresis and transfer to a membrane, or dot protein samples onto the membrane as described in the “Signal capture” section.

2. Cut strips from the membrane according to the number of conditions being tested. The following combinations should be tested with each blocker:

   - Blocker + primary antibody + enzyme conjugate + substrate

   - Blocker + enzyme conjugate + substrate

   - Blocker + substrate

3. Add the strips to various blocking solutions, ensuring the strip is completely immersed in the solution. Incubate each strip for 1 hr at room temperature with shaking.

4. Add primary antibody containing 10% blocking agent (v/v) to appropriate groups containing strips with blocking solution. Other groups will remain in the blocking solution until it is time to expose to another solution. If optimized dilutions have not yet been determined, use a mid-range value according to the sensitivity of the substrate.

5. Add enzyme-conjugate solution containing 10% blocking agent (v/v) to all groups except the one containing the blocker and substrate. If optimized dilutions have not yet been determined, use a mid-range value according to the sensitivity of the substrate.

6. Wash membrane and add the substrate. Image the blot as desired.

Optimizing the primary antibody concentration

1. Separate the protein sample by electrophoresis and transfer to the membrane. Alternatively, dot the protein sample onto the membrane as described in the section for optimizing antigen concentration. Block the membrane using an appropriate blocking reagent. Cut strips from the membrane according to the number of primary antibody conditions being tested.

2. Prepare dilutions of primary antibody in wash buffer containing 10% blocking agent (v/v) and apply to the membrane strips. Incubate for 1 hr at room temperature.

3. Wash the strips and incubate with enzyme conjugate for 1 hr at room temperature. Wash again and develop signal using an appropriate substrate. Image the blot as desired.

Optimizing membrane washing

1. Use a wash buffer such as PBS or TBS or other physiological buffer containing 0.05% Tween 20 detergent.

2. Wash the membrane by agitating at least 3 times for 5 min each after primary antibody incubation, and at least 5 times for 5 min each after incubating with the enzyme conjugate.

3. If nonspecific background appears upon final detection, use larger volumes of wash buffer or increase the number and time of each wash. If no improvement occurs, the problem lies with another variable.

Optimizing enzyme conjugate concentration

When determining the optimal concentration for a new western blotting system, a simple experiment often saves much frustration with potential signal variability.

1. Apply the same amount of target in three (or more) wells of the gel.

2. Separate the protein sample by electrophoresis and transfer the sample to the membrane. Block the nonspecific binding sites and probe with the primary antibody.

3. After washing, cut the blot into strips containing the target.

4. Probe each strip with a different concentration of the secondary antibody conjugate. For example, for SuperSignal West Pico Plus Substrate use 1:40,000, 1:60,000, and 1:80,000 dilutions (from a 1 mg/mL stock). Incubate strips for 1 hr at RT with continuous rocking.

5. Wash the strips and add the substrate. After substrate incubation, image the strips.

6. Wait 1–2 hr and image the strips a second time.

7. Evaluate the results. For example, if the second exposure results in a signal for the 1:80,000 dilution and the signals from the other two dilutions are faint upon second imaging, then the 1:80,000 dilution is the closest to the optimum. But if the 1:40,000 dilution has a strong signal and the other two dilutions produce faint signals, then the more concentrated dilution is closer to the optimum.

Optimizing the detection method

1. Separate the protein sample by electrophoresis and transfer to membrane, or dot the protein sample onto the membrane as described in “Optimization of antigen concentration” section.
2. Block nonspecific binding sites and probe with the primary antibody and enzyme conjugate containing 10% blocking agent (v/v).
3. If antibody concentrations have not been optimized, choose a mid-range value. Wash the membrane after each incubation.
4. Cut strips from the membrane according to the number of substrate exposure conditions being tested. Prepare working solution of the substrate to be tested.
5. Incubate the membrane strips with the substrate for a time period consistent with the manufacturer’s instructions.
6. Remove the strips from the substrate using forceps and gently tap edge onto a paper towel to remove excess substrate.
7. Place the strips in a plastic cover and image the blot for varying lengths of time. Select a time that shows clear signal and low background.

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SuperBlock T20 (TBS) Blocking Buffer	1 L	37536
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Pierce Protein-Free (TBS) Blocking Buffer	1 L	37570
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