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Detailed Western Blotting (Immunoblotting) Protocol

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Western Blotting, which is probably better referred to as immunoblotting, is one of the most commonly used biological methods worldwide. This technique is capable of detecting an individual protein from a complex mixture of proteins extracted from cells or tissues. The major steps in the Western Blotting workflow are 1) The separation of proteins based on size, 2) The transfer of separated protein to a suitable stable support, 3) Interaction between the target protein and appropriate primary antibodies. In many cases, a secondary antibody that interacts with the primary antibody is used, and 4) Visualization of the target protein using enhanced chemiluminescence (ECL), fluorescence, or colorimetric methods. A detailed protocol to help users get the most out of their Western Blots is presented.

Goal: To help Western Blotting users learn what prevents them from having perfect Western Blots

The most common result of your experiment:

A good Western blot.

Another possible result:

No signal detected or weak signal

Most likely reasons:

1. Antibody was not suitable (poor quality antibody).
2. Insufficent protein loaded on the gel for the amount of antibody used (or antibody too dluted).
3. Transfer efficiency from gel to membrane was poor.
2. ECL reagent was expired or contaminated.
5. Incorrect secondary antibody used.
6. Blocking agent concentration was too high.

For more reasons read WESTERN BLOTTING TIPS AND TROUBLESHOOTING GUIDE

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protocol

Rajeshwary Ghosh, Jennifer E Gilda and Aldrin V Gomes. (2014) The necessity of and strategies for improving confidence in the accuracy of western blots. Expert Review of Proteomics 11(5): 549-560.

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The number of companies selling products for Western Blotting continues to increase. However, no reports of any company selling harmful Western blotting products has been reported. As such, feel free to use products from any company, except antibodies. We recommend searching for antibody companies and reviews as well as for the specific antibody and reviews as some companies' antibodies have been reported to be substandard. The most common cause of Western blotting problems is due to the primary antibody used.

Laemmli 2X buffer/loading buffer

- 4% SDS
- 10% 2-mercaptoethanol
- 20% glycerol
- 0.004% bromophenol blue
- 0.125 M Tris-HCl

Check the pH and adjust to 6.8

Running buffer (Tris-Glycine/SDS)

- 25 mM Tris base
- 190 mM glycine
- 0.1% SDS

Check the pH and adjust to 8.3

Transfer buffer (wet)

- 25 mM Tris base
- 190 mM glycine
- 20% methanol

Check the pH and adjust to 8.3

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Transfer buffer (semi-dry)

- 48 mM Tris
- 39 mM glycine
- 20% methanol
- 0.04% SDS

Blocking buffer

- 5% BSA (bovine serum albumin) made with TBST, or
- 3% milk made with TBST

Mix well. If you see spotting of blocking protein (dark spots seen during color development) on the membrane, filter the solution before use.

Tris-buffered saline containing Triton X-100 (TBST)

Make a 10X solution of Tris-buffered saline (TBS)

- 200 mM Tris base
- 1.5 M NaCl

Check the pH and adjust to 7.5.

If you notice your TBS solution getting cloudy after a few days/weeks, then autoclave your TBS to sterilize it.

1X solution of TBST

- 100ml of 10X TBS stock solution
- 1ml of Tween 20 detergent
- 899 ml of Milli-Q water

Mix well, then mix each time before use.

Pre-stained standard commonly used:

Bio-Rad Precision Plus protein Standard by Bio-Rad Laboratories
Catalog #161-0374

Chemiluminescence reagent commonly used:

Clarity Western ECL Substrate by Bio-Rad Laboratories
Catalog # 1705061

Blocking Reagent

Blotting Grade Blocker Non Fat Dry Milk by Bio-Rad Laboratories
Catalog # 1706404XTU

Membranes

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Immun-Blot PVDF Membrane, Roll, 26 cm x 3.3 m by Bio-Rad Laboratories
Catalog #1620177

Nitrocellulose Membrane, Roll, 0.2 µm, 30 cm x 3.5 m by Bio-Rad Laboratories
Catalog #1620112

Immun-Blot® Low Fluorescence PVDF/Filter Paper Sets by Bio-Rad Laboratories
Catalog #1620260

Acrylamide is a neurotoxin that can be absorbed not just orally but through the skin and by inhalation. Hence, if you are pouring your gels, use caution. Always use appropriate personal protective equipment (PPE), gloves, eye protection and lab coats.
While polymerized acrylamide is non-toxic and can be disposed of in the trash, the unpolymerized liquid is hazardous waste. Label unpolymerized acrylamide as Hazardous Waste and accumulate according to your company or institution's requirements.

Although the western blotting procedure is straightforward and well documented, problems can arise, leading to unexpected results.
A significant cause of problems is poor quality samples. For example, if samples were not stored properly before denaturation in sample buffer, many proteins may be proteolyzed and give artifactual results. Invest the time to ensure your samples are well prepared because, even if you do every other part of Western blotting perfectly, poor quality samples will result in inaccurate Western blots.

Sample preparation

1 Sample preparation can be simple or complex, depending on the source of the sample and the location of the target protein. It is recommended that you consult a dedicated article with procedures for optimal sample preparation.
This protocol assumes you have already prepared your sample and are ready to begin mixing with gel electrophoresis sample buffer.

1.1 Remove a small volume of sample to perform a protein quantification assay. Determine the protein concentration for each cell sample. Use a protein quantification appropriate method that is compatible with the reagents in your lysis/homogenization buffer.

No individual protein assay method is compatible with all the different sample components used in a typical research laboratory.

The most common methods for quantitation of protein involve either protein-dye binding chemistry (Coomassie reagent/Bradford assay) or protein-copper chelation chemistry (bicinchoninic acid (BCA) and Lowry

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assays). For some samples, the absorbance of the sample at 280nm could be used.

A comprehensive list of reagents and their compatibility with different protein assays can be found here:

Protein Assay Compatibility Table

1.2 Determine how much protein to load in each well and how many wells you will load. If you are going to load many wells on different gels over several weeks then prepare them at the same time. Once you know how much sample you need, dilute your sample appropriately or use it as is and add an equal volume of 2X Laemmli sample buffer to get the final concentration of your protein at a concentration you want. We recommend reducing and denaturing the samples unless the online antibody datasheet indicates that non-reducing and non-denaturing conditions should be used.

Instead of the Laemmli sample buffer, you can use a Dual Color Protein Loading Buffer (Dual Color Protein Loading Buffer 4X by Boster Bio, Catalog #AR1142) that contains 2 tracking dyes, Bromophenol Blue, and Pyronin Y. The bromophenol blue is colored blue and tracks the progress of proteins during electrophoresis, while the pink Pyronin Y is useful for tracking the protein transfer to the membrane.

1.3 To reduce and denature your samples, heat each cell lysate in sample buffer at 75°C for 5 mins. Allow samples to cool and then use or aliquot and store at -20°C for future use (good for at least three months). For longer storage store at -80°C.

While many reports suggest that it is safe to store denatured samples at 4°C indefinitely, we have found that a few proteins start degrading after 4 weeks so our laboratory does not keep denatured samples at for more than 2 weeks.

Avoid repeated freeze-thaw cycles of denatured proteins. Consider aliquoting small amounts if you plan to run gels a few months apart.

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Important: A common mistake is heating samples at 100°C for 5 mins. Prolonged heating of proteins at high temperatures (95-100°C for 5 mins) results in cleavage of the Asp-Pro protein bonds (Kurien BJ and Scofield RH, 2012). Heating at 75°C for 5 min avoids Asp-Pro bond cleavage, but still inactivates proteases and denatures proteins. A few proteins are stable to heat (even 100°C for several hours).

Heating the samples serve two main purposes:
it allows better sample denaturation,
it inactivates proteolytic enzymes in the sample

Loading and Running the Gel

2 Loading the sample is important as errors in loading will result in inaccurate results. Prepare the electrophoresis tank by placing the gel inside the tank and fill with SDS-PAGE running buffer. Use the same pipette for loading all the wells and if the amount loaded should be the same then ensure that the level of the sample in each lane is the same by eye.

2.1 Load a molecular weight marker in appropriate wells of the SDS-PAGE gel, such as lane 1. Bio-Rad Precision Plus protein Standard Catalog #161-0374 (Bio-Rad Laboratories) works well.

Precision Plus Protein Dual Color Standards contain a mixture of 10 recombinant proteins (10–250 kD), 8 blue-stained bands, and 2 pink reference bands (25 and 75 kD). The colored bands are especially helpful in determining the molecular mass of target proteins and is also useful for the orientation of your blot.

2.2 We typically load 8-15 µg of total protein for cell lysates or tissue homogenates or 10–100 ng for purified protein. Larger amounts of protein can result in non-linearity when housekeeping proteins are used as normalization standards.

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2.3 Run the gel for 1–2 hr at 120 V until the dye front reaches the agarose layer of the gel. Depending on the gel system you can run at higher voltages (consult your equipment manufacturer's guide).
When the dye front (caused by the bromophenol blue in the Laemmli buffer) reaches near the bottom or at the bottom of the gel, remove the gel from the glass or plastic plates. It is recommended that the wells be removed with a razor blade or similar object as this helps reduce your chance of having bubbles during transfer and makes handing of the gel easier.
Make a small cut at the top left side of the gel for orientation.

Note: The time and voltage require optimization. We recommend following the manufacturer's instructions. A reducing gel should be used unless non-reducing conditions are recommended on the antibody datasheet.

2.4 The gel percentage required is dependent on the size of your protein of interest:

A	B
Protein size	**Gel percentage**
4–40 kDa	20%
12–45 kDa	15%
10–70 kDa	12.5%
15–100 kDa	10%
25–100 kDa	8%
60-210 kDa	5%

Gel Percentage and Estimated Optimal Protein Separation Range

Gradient gels can also be used.

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A	B
Protein size	**Gel percentage**
5-200 kDa	4-12 gradient
4-200 kDa	4-20 gradient
3.5-110 kDa	10-20 gradient

Gel Percentage and Estimated Optimal Protein Separation Range

The estimated protein separation range depends on many factors including the running buffer used (Tris/Glycine, MOPS, MES), the sample buffer used, and additives that may have been added to the acrylamide gel.

If you have problems with gel electrophoresis, consult this:
World's Most Detailed Gel Electrophoresis Troubleshooting Guide

Transferring the protein from the gel to the membrane

3 The membrane can be either nitrocellulose or polyvinylidene fluoride (PVDF). There are a few differences between the two membranes:

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A	B
Nitrocellulose	PVDF
Less Expensive	More Expensive
Fast Protein Binding	Higher Sensitivity Protein Binding
Does Not Require Pre-Wetting	Requires Pre-Wetting with Methanol
Weaker Than PVDF	Stronger Than Nitrocellulose
Not Recommended for Stripping and Reprobing	Recommended for Stripping and Reprobing
Lower Protein Binding Capacity Than PVDF	Good for Hydrophobic Proteins
	Requires Re-wetting with Methanol Throughout Use

Advantages and disadvantages of the two most commonly used membranes for Western Blotting.

3.1 Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step.

3.2 If using a wet or tank transfer, soak one stack of filter paper in transfer buffer and place it on the sponge. Wet the nitrocellulose membrane with transfer buffer and lay it on top of the filter paper stack. Allow at least 2 min for the filter paper and membrane to soak in the transfer buffer. Place the gel on top of the membrane, followed by another stack of filter paper soaked in transfer buffer.
Firmly roll out any air bubbles with a roller (such as a clean pencil). Use your fingers to apply mild pressure on the whole transfer stack while closing the cassette to ensure no additional air bubbles form. Once the stack has been completed, place the stack into the tank and fill the tank with transfer buffer. The tank should be run between 0.1 to 1 amps or 5 to 30 V for an hour or overnight, depending on the amp or voltage.

The time and voltage of transfer may require some optimization. We recommend following the manufacturer's instructions.

Using cold transfer buffer helps prevents overheating of the gel.

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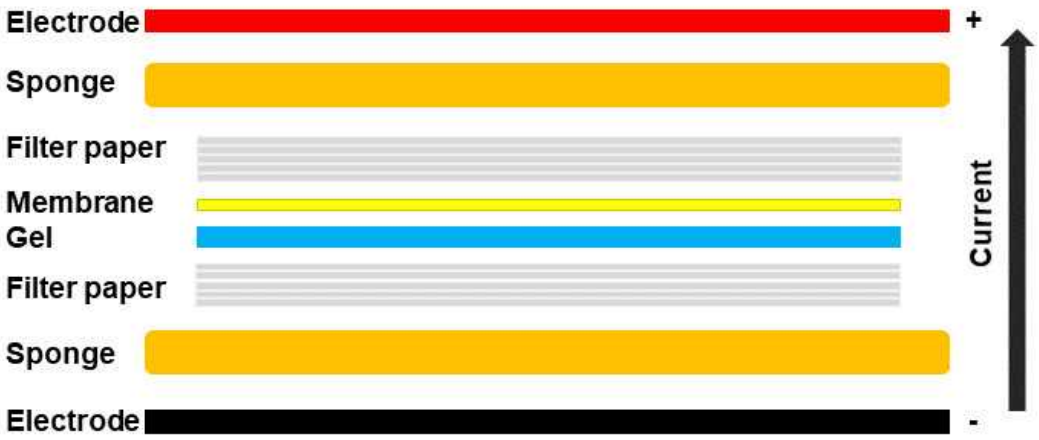
3.3 If using the Trans-blot Turbo (fast transfer), soak one stack of filter paper in transfer buffer and place it in the Trans-Blot Turbo Transfer System cassette. Wet the nitrocellulose membrane with transfer buffer and lay it on the filter paper stack. Allow at least 2 min for the filter paper and membrane to soak in the transfer buffer. Place the gel on the membrane, followed by another stack of filter paper soaked in transfer buffer. Firmly roll out the air bubbles with a roller. Use your fingers to apply mild pressure on the whole transfer stack while closing the cassette lid to ensure no additional air bubbles form. Wipe the outside of the cassette with tissue paper and place it inside the Trans-Blot Turbo Transfer System machine. Run with the appropriate setting. We use midi gels so our transfer uses the Turbo midi setting of 7 min to transfer the proteins from the gel to the membrane.

3.4 Prepare the stack as follows:



3.5 Take the membrane from the cassette and image it while still wet in the ChemiDoc MP Imaging System. If using Stain-Free gels, then use the Stain-Free membrane setting and optimized exposure time. There is no need to activate the membrane again. Adjust the exposure time as needed to avoid overexposed images. If necessary, the membrane can be cut into smaller sizes at this stage. If using normal gels use Ponceau S to stain the membranes.

Different methods are available to stain the protein on the membrane. For determining if the transfer was efficient Amido Black and Ponceau S staining is quick and cheap.

0.1% Amido Black Solution with shaking for a few minutes until the bands appear. Remove Amido Black by washing membrane in distilled water three times, 5 minutes each, with mild agitation.

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We use Ponceau S. The relatively inexpensive 0.01% Ponceau S in 1% acetic acid stain for total protein normalization works well. Incubate your membrane in Ponceau S for 2 minutes and then rinse briefly for a few seconds two times. Image your blot to use this as your normalization when quantifying your blot. More information is available here. Remove ponceau stain with TBS (2 1 minute washes) or water (3 one minute washes).

3.6 Dry the membrane on top of a piece of tissue paper. Allowing the membrane to dry before proceeding to immunostaining has been suggested to help the proteins adhere better to the membrane.

Conflicting reports of how to store membranes and for how long are available on the internet.

This is our experience:

After drying, keep the membrane between two clean sheets of thin cardboard. You can use paper clips to clip the stack together. Store the membrane in a zip lock bag at 4˚C for up to 2 weeks. Membranes can also be stored at -20˚C for up to 3 months, or -70˚C for longer storage.

Blocking

4 Blocking is an important step in the Western blotting process. Blocking prevents the non-specific binding of antibodies to the membrane.

4.1 Place the membrane in a blotting container and add enough blocking solution (3% Milk in TBST, or 5% BSA in TBST) to cover the entire membrane surface. Pour solutions into the corner of the blotting container and not directly onto the membrane so that the proteins on the membranes are not disturbed. Incubate the membrane on a shaker for 1 h. Pour out the blocking solution and rinse one time with TBST for 10-20 seconds to remove excess milk.

Blocking is often done with 5% BSA or nonfat dried milk diluted in TBST to reduce the background. Nonfat dried milk is usually preferred as it is inexpensive and widely available. We found that 3% nonfat milk worked

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just as great for reducing the background noise while enhancing the target protein signal.

Blocking the membrane for longer than 1 hour at room temperature also gave lower band intensities.

Some studies have shown that cheap and disposable plastic wear similar to what many labs use for incubating their Western blots can leach chemicals that can have effects on their results.
Simply washing the plastic with methanol removes most of these chemicals. At the very least, wash all new plasticware two times with distilled water before use. This also removes some of the "leachable chemicals."

If phosphorylation-specific antibodies are used, the blocking solution (and antibody solutions) should not contain milk since milk contains phosphoproteins.

Primary Antibody Staining

5 ⚠

The most critical part of any Western blot is the primary antibody.

5.1 Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer (1% Milk in TBST, or 3 % BSA in TBST). We recommend overnight incubation at 4°C, or at least 4 hours incubation at room temperature.

Most laboratories use 5% Milk in TBST, or 5% BSA in TBST for incubating antibodies. We have found that that decreases the final signal obtained. Just 1% nonfat milk was enough to give great results.

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You can learn about antibody concerns and problems with Western blotting in general by watching our lab videos.

Can we trust Western Blots?
Western blotting: not as easy as it looks

Limitations of Housekeeping Proteins

The use of housekeeping proteins for reproducible normalization requires the resulting blot signals to be within a linear range, and the expression of the housekeeping protein to not vary with the experimental conditions being investigated. We recommend using total protein staining as a control (either Ponceau S or Stains free staining).

Washing

6 Although it may seem like a trivial step, washing is important for improving the signal to noise (background) so that quantification is more accurate.

6.1 Wash the membrane in three washes of TBST, 3 min each.

If you notice high background with some antibodies, increase each wash step's length to 5 minutes. In our hands, we need to do this less than 10% of the time. For most antibodies, three 5 minute wash steps reduce the final signal intensity/noise ratio we get.

If you purchase TBS, ensure you check the composition of the TBS. Some companies have different concentrations of Tris and NaCl. We once purchased TBS from a company and none of our Westerns worked. The company had 500 mM NaCl instead of 150 mM NaCl.

Secondary Antibody

7 The choice of companies to purchase secondary antibodies is huge. Some companies have

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well established secondary antibodies that are respected and work well. We have used secondary antibodies from Cell Signaling, Bio-Rad, Sigma, and Abcam and they all worked well.

7.1 Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer (1% Milk in TBST) at room temperature for 1 hr. For chemiluminescence, the secondary antibody is conjugated with horseradish peroxidase (HRP).

Washing Part II

8 Time to remove excess secondary antibodies.

8.1 Wash the membrane in three washes of TBST, 3 min each.

Detection

9 The choice of signal development reagents is expanding yearly. Most commercial preparation work well. Of 21 different commercial products we have tried, only one was disappointing.

9.1 For signal development, follow the kit manufacturer's recommendations the first time you use their reagents.

9.2 Because of price and sensitivity, we utilize Clarity enhanced chemiluminescence (ECL) reagent. The amount of ECL reagent depends on the size of the blot. For a 3 cm x 4 cm membrane strip, we use 1 ml of ECL reagent. In a 1.5 mL centrifuge tube, mix equal parts of Clarity western peroxide agent (0.5 mL) and Clarity western luminol/enhancer reagent (0.5 mL). This creates the substrate for chemiluminescence. Pipette enough ECL substrate to evenly cover the surface of the membrane where the proteins are attached. Incubate the ECL covered membrane in a dark condition for 2 min before removing the excess ECL reagent.

The optimal incubation time to incubate an ECL reagent should be determined with each new ECL reagent. Try simple experiments to determine what incubation time with the detection reagent works best for your lab. Sometimes shorter or longer incubations than the manufacturer suggest work a lot better.

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Imaging

9.3 Pick up the membrane from one side with a pair of blunt forceps and drain the excess ECL reagent by gently touching the edge of the membrane to a delicate tissue paper. Arrange the membrane on the imaging surface, taking care not to create bubbles on the surface.

9.4 Image the blot on your machine of choice. We utilize the Bio-Rad ChemiDoc MP Imaging system with the Chemi Hi Sensitivity setting and 10 seconds of imaging time initially. If the image is saturated (red bands) then we use the Chemi Hi-Resolution setting with the optimized exposure time. If the initial 10 s image was not visible, we do a 60 s high sensitivity image.
The exposure time can be adjusted as needed to obtain the best image that contains no overexposed (saturated) bands.
One of the advantages of the ChemiDoc MP is that we are able to take a multichannel image with the stain-free and either the Chemi Hi Sensitivity or Hi-Resolution setting to get an image of the chemiluminescent protein bands of interest overlaid with the stain-free prestained protein markers.
Although the other proteins on the membrane will also show up with the stain-free image, the chemiluminescent signal shows up on a different channel.
Verify the molecular weight of the band of interest based on the multichannel image.

If the signal is weak, the ECL reagent can be left for longer than 2 min, but when the ECL reagent is left on the membrane for longer than 5 min, the length of time that the maximum signal lasts is shorter than when the ECL reagent is left for 2 min. Therefore, we recommend removing the excess ECL reagent and leaving the ECL reagent on for no more than 5 min (however, this will depend on your specific ECL reagent used).

10 After you get your great looking blots you may want to quantify your results.
A major concern of western blot quantification is the difficulty associated with determining saturated signals. This is especially problematic when using film. It is recommended that a digital imager be used (many universities now have digital imagers so try to find a lab that has one). Please read the manufacturer's recommendation on how to use the specific imager you are using as well as do a google search for problems with quantification using the software that you will use to quantify. Unfortunately, after getting great blots some researchers do inaccurate quantification of their results. Invest your time to learn how to quantify the signals accurately.

Relax

11 Sit back and enjoy your Western blotting results.

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If you want super sensitivity for your Western blots consider using the technique presented in our paper:
Mishra M, Tiwari S, Gunaseelan A, Li D, Hammock BD, Gomes AV. (2020)Improving the sensitivity of traditional Western blotting via Streptavidin containing Poly-horseradish peroxidase (PolyHRP).Electrophoresis, 40(12-13): 1731-1739.

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